

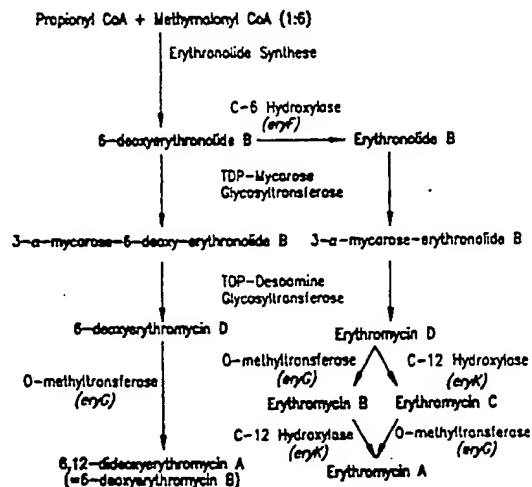
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : C12N 15/76, 15/90, 9/10, C12P 19/62</p>	<p>A1</p>	<p>(11) International Publication Number: WO 97/06266</p>	<p>(43) International Publication Date: 20 February 1997 (20.02.97)</p>
<p>(21) International Application Number: PCT/US96/12850</p> <p>(22) International Filing Date: 2 August 1996 (02.08.96)</p> <p>(30) Priority Data: 60/001,835 3 August 1995 (03.08.95) US</p> <p>(71) Applicant: ABBOTT LABORATORIES [US/US]; CHAD 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).</p> <p>(72) Inventors: STASSI, Diane, L.; 1883 Elmwood Drive, Highland Park, IL 60035 (US). MAINE, Gregory, T.; 5743 Constitution Avenue, Gurnee, IL 60031 (US). POST, David, A.; 4914 Harrison, Kenosha, WI 53142 (US). SATTER, Mark; 7099 61st Avenue, Kenosha, WI 53142 (US).</p> <p>(74) Agents: DANCKERS, Andreas, M. et al.; Abbott Laboratories, CHAD 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).</p>		<p>(81) Designated States: CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published With international search report.</p>	

(54) Title: VECTORS AND PROCESS FOR PRODUCING HIGH PURITY 6,12-DIDEOXYERYTHROMYCIN A BY FERMENTATION

(57) Abstract

A process for producing high purity 6,12-dideoxerythromycin A using recombinant DNA technology is disclosed. The erythromycin producing strain, *Saccharopolyspora erythraea*, lacking the erythromycin C-12 and C-6 hydroxylases produces a mixture of 6,12-dideoxerythromycin A and the precursor molecule, 6-deoxyerythromycin D. To achieve conversion of the precursor to the final product, a second copy of *eryG* is inserted into a non-essential region of the *Sac. erythraea* chromosome resulting in high purity 6,12-dideoxerythromycin A.

[illegible]

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

**VECTORS AND PROCESS FOR PRODUCING HIGH PURITY 6,12-
DIDEOXYERYTHROMYCIN A BY FERMENTATION**

5 This application claims the benefit of U.S. Provisional Application No. 60/123,456,
filed June 8, 1995.

TECHNICAL FIELD

10 The present invention relates to the production of an erythromycin derivative. In
particular, the present invention relates to the production of high purity 6,12-
dideoxyerythromycin A through genetic manipulation of the producing organism.

BACKGROUND OF THE INVENTION

15 Erythromycin A is a clinically useful, broad-spectrum macrolide antibiotic produced
by the gram positive bacterium, *Saccharopolyspora erythraea* (*Sac. erythraea*) Intermediates
of erythromycin biosynthesis, which may be useful in the design and development of new
drugs, are produced in minute quantities by *Sac. erythraea* and occur as mixtures with other
20 erythromycin derivatives, complicating chemical modifications of these compounds.

 As taught in the art, (see Donadio et al., *Genetics and Molecular Biology of Industrial
Microorganisms*, eds. C. L. Hershberger, S. W. Queener, and G. Hegeman, 1989, American
society for Microbiology, Washington, DC 20005) the biosynthesis of erythromycin A by
Sac. erythraea, is achieved according to the proposed right-hand pathway shown in FIG. 1.
25 The 14-membered macrolactone, 6-deoxyerythronolide B, is first made from propionyl and 2-
methylmalonyl thioesters and is then hydroxylated at the C-6 position to form erythronolide
B. The sugars mycarose and desosamine are synthesized from glucose and are added to
erythronolide B to make erythromycin D. The next steps in the proposed pathway are (in
either order) the hydroxylation of erythromycin D at the C-12 position (resulting in the
30 formation of erythromycin C) or methylation of the C-3" position (resulting in the formation
of erythromycin B). Subsequent hydroxylation of erythromycin B or methylation of
erythromycin C produces erythromycin A.

 Our present understanding of the genes responsible for the biosynthesis of
erythromycin and techniques to inactivate genes in *Sac. erythraea* allow the directed
35 manipulation of the pathway in order to produce precursors and derivatives of erythromycin
A. Naturally occurring precursors of erythromycin A, such as erythromycin B and
erythromycin D are readily produced by these methods. However, other attempts to make
highly pure derivatives of erythromycin A *in vivo* are not always successful, especially when

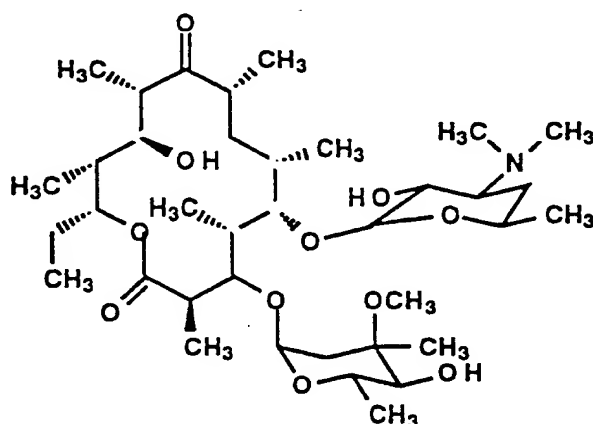
- 2 -

alterations are made which change the substrates for enzymes acting in later stages of biosynthesis. It is in these cases where further genetic modifications may become necessary.

SUMMARY OF THE INVENTION

The method of the present invention includes the genetic modification of an erythromycin producing microorganism, so that it is transformed into a strain which produces high purity 3"-O-methylated erythromycin derivatives. In particular, a non-essential region of the chromosomal DNA is genetically modified by the insertion of a second copy of *eryG*, whose product is the erythromycin 3"-O-methyltransferase, and which normally converts erythromycins D and C into erythromycins B and A, respectively.

A microorganism embodying the present invention is a novel strain of *Sac. erythraea* which, upon cultivation in an aqueous medium, produces high purity 6,12-dideoxyerythromycin A of the formula:



[1]

Transformation of an erythromycin-producing microorganism into a 6,12-dideoxyerythromycin A producing strain is accomplished by mutagenic techniques, and in particular, through gene replacement by homologous recombination. Using this methodology, the *eryF* and *eryK* genes, which encode the cytochrome P-450 enzymes essential for hydroxylating erythromycin at the C-6 and C-12 positions, respectively, are replaced by integrative plasmids which carry deletions in these genes. As a result of replacing the wild type genes with the deleted copies, neither the C-6 nor C-12 positions are hydroxylated. As shown theoretically in the left hand side of FIG. 1, a deletion in the *eryF* gene prevents the conversion of 6-deoxyerythronolide B to erythronolide B; the addition of the sugar groups results then in the formation of 6-deoxyerythromycin D. The second deletion mutation, i.e. in the *eryK* gene, prevents hydroxylation of 6-deoxyerythromycin D to 6-deoxyerythromycin C. Thus, in the absence of a functional *eryK* gene, methylation of 6-

- 3 -

deoxyerythromycin D results directly in the formation of 6,12-dideoxyerythromycin A (which may also be designated as 6-deoxyerythromycin B).

However, a complicating factor in the formation of a 6,12-dideoxyerythromycin A producing strain is that 6-deoxyerythromycin D serves a poor substrate for the erythromycin 3"-O-methyltransferase, which converts the substrate to 6,12-dideoxyerythromycin A. This results in a low ratio of the desired 6,12-dideoxyerythromycin A product to 6-deoxyerythromycin D, the precursor. Thus an additional requirement for the production of high purity 6,12-dideoxyerythromycin A is the introduction of a second copy of the gene, *eryG*, which encodes the 3"-O-methyltransferase, into the producing organism. In this particular embodiment of the invention, a plasmid was constructed which allowed a second copy of *eryG*, driven by the *ermE** promoter, to be inserted via homologous recombination into a non-essential region of the *Sac. erythraea* chromosome and to be stably maintained in the *Sac. erythraea* strain.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be more readily appreciated in connection with the accompanying drawings, in which:

FIG. 1 is a proposed metabolic pathway for the biosynthesis of erythromycin A (on the right-hand side) and 6,12-dideoxyerythromycin A in *Sac. erythraea*; (on the left-hand side);

FIG. 2 is a flow diagram depicting the construction of pDPE4;

FIG. 3 is a flow diagram depicting the construction of pGM504;

FIG. 4 is a flow diagram depicting the construction of pDPE35;

FIG. 5 is a schematic representation of gene replacement in *Sac. erythraea*;

FIG. 6 is the thin layer chromatography of the products of the fermentation of ER720-KF;

FIG. 7 is the thin layer chromatography of the products of the fermentation of ER720-KFG+;

FIG. 8a illustrates the amounts of 6,12-dideoxyerythromycin A and 6-deoxyerythromycin D produced in a genetically engineered *Sac. erythraea* strain ER720-KF;

FIG. 8b illustrate the amounts of 6,12-dideoxyerythromycin A and 6-deoxyerythromycin D produced by the genetically engineered *Sac. erythraea* strain ER720-KFG+;

FIG. 9 is a flow diagram depicting the construction of pKAS37.

FIG. 10 depicts a restriction map of pKAS37.

FIG. 11 is a flow diagram depicting the construction of pKASI37.

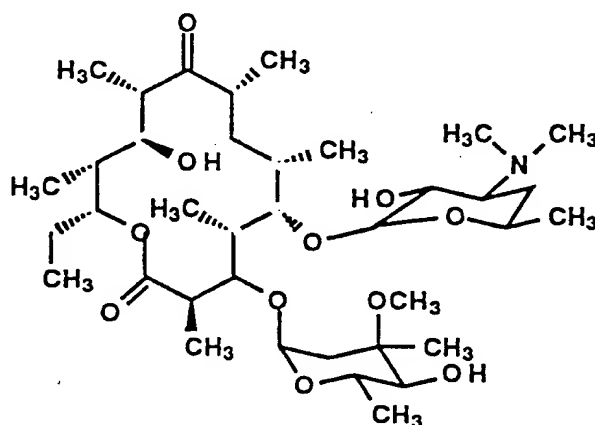
FIG. 12 depicts a restriction map of pKASI37.

- 4 -

FIG. 13 depicts the single stranded DNA sequence of a fragment of DNA contained within the second site region.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the genetic modification of erythromycin producing microorganisms which enables them to produce highly pure erythromycin derivatives having -O-methylation at the 3" position of the molecule. The compounds of the present invention include 6,12-dideoxyerythromycin A which is represented by the structural formula:



[1]

This compound was obtained by growing the genetically modified erythromycin producing microorganism in liquid culture and then extracting the compound from the culture medium; the compound was found to be the dominant erythromycin derivative in the fermentation.

The present invention provides a method for the preparation of the highly pure 3"-O-methylated erythromycin derivatives, which comprises transforming an erythromycin producing strain into a variant producing the desired compound. In one embodiment of the invention, the erythromycin producing microorganism is the bacterium, *Sac. erythraea*. Following genetic manipulation, the resulting transformant is not only deficient in the cytochrome P-450 enzymes encoded by *eryF* and *eryK* but also contains an additional copy of *eryG*, which encodes the erythromycin 3"-O-methyltransferase. It is the presence of this second copy of *eryG* which allows efficient conversion of 6-deoxyerythromycin D to 6,12-dideoxyerythromycin A, and in so doing, provides a strain of *Sac. erythraea* which produces high purity end product rather than a mixture of erythromycins as shown in FIGS. 8a and 8b.

The present invention also provides, as an example, a particular method for the introduction of the second copy of *eryG* into a non-essential region of the *Sac. erythraea*

- 5 -

chromosome, comprising replacement, by homologous recombination, of a section of that non-essential region of the chromosome with a copy of the same region containing embedded therein a thiostrepton resistance marker and a second copy of *eryG* which is under the control of the *ermE** promoter.

5 The methods of the present invention are widely applicable to erythromycin-producing microorganisms, including but not limited to *Saccharopolyspora* species, *Streptomyces griseoplanus*, *Nocardia* sp., *Micromonospora* sp., *Arthrobacter* sp. and *Streptomyces antibioticus*. Of these, *Sac. erythraea* is the most preferred. Of course, the specific sequence of the homologous second site in a non-essential region of a different microorganism may
10 vary somewhat from that shown in SEQ. ID. NO.:1 for *Sac. erythraea* but the method of identifying such a site is within routine skill of those practicing the art.

Both the C-6 and C-12 hydroxylations are catalyzed by cytochrome P-450 enzymes encoded for by the *eryF* and *eryK* genes, respectively. An erythromycin-producing *Sac. erythraea* strain which lacks these two activities would be predicted to produce 6,12-
15 dideoxyerythromycin A. One means of eliminating these hydroxylation reactions is through a disabling mutation of the cellular genes required for the operation of the cytochrome P-450 monooxygenase system. This can be accomplished by replacing these genes with copies containing deletions, thereby making the genes non-functional and non-revertable. Any plasmid designed for gene replacement by homologous recombination which disrupts the
20 hydroxylation steps in erythromycin biosynthesis can be utilized. Furthermore, the method of the present invention is in no way limited to the use of gene replacement to produce mutants defective in C-6 and C-12 hydroxylation of erythromycin. Other systems which disrupt the hydroxylase systems, such as gene disruption, transposon mutagenesis or chemical or light induced mutagenesis, can be used to produce the desired genetic modification of the
25 microorganism. Such alternative procedures are well known to those of ordinary skill in the art.

Although several methods are known in the art for inserting foreign DNA into a plasmid to form a gene replacement plasmid, the method preferred in accordance with this invention is shown schematically in Figs. 2 and 3 and demonstrated in the Examples below.
30 In a preferred embodiment of the present invention, selectable DNA plasmids are constructed which comprise (a) a fragment of plasmid pIJ702 or pIJ486 containing an origin of replication and a fragment of DNA conferring resistance to the antibiotic thiostrepton (*tsr*), each of which are functional in *Streptomyces*; (b) an origin of replication and a DNA fragment conferring resistance to the antibiotic ampicillin (*amp*), each of which are functional in *E. coli*, and (c) a
35 DNA fragment from the *Sac. erythraea* chromosome containing the mutated (i.e. deleted) gene of interest and at least about 1 kb of contiguous DNA flanking both sides of the mutated gene, each of which is capable of acting as a recognition sequence for plasmid integration and subsequent excision of the plasmid from the genome. If the excision event occurs on the side

- 6 -

of the deletion opposite of that of the integration event, the wild type gene will be replaced by the deleted one, as schematically illustrated in FIG. 5. Example 1 and FIG. 2 are examples of a plasmid constructed for creating a deletion in *eryK*. Example 2 and FIG. 3 are examples of a plasmid constructed for creating a deletion in *eryF*.

5 The particular antibiotic resistance genes and functional origins of replication identified above are necessary only inasmuch as they allow for the selection and replication of the desired recombinant plasmids. Other functional markers and origins of replication may also be used in the practice of the invention. Likewise, any recognition sequence may be used which enables the recombinant plasmid to be integrated into a portion of the genome adjacent
10 to the gene of interest and excise on the other side of the gene of interest in order to replace that gene with a mutated copy. In addition, the plasmid of the invention may be constructed without the use of a partial genomic digest, as in the above examples. Instead, if the sequences of the regions flanking *eryF* and *eryK* are known, a recognition sequence may be synthesized de novo (for example by polymerase chain reaction) and ligated with the
15 necessary origin and resistance fragments to form the gene replacement plasmids.

 In Example 4, an erythromycin producing strain of *Sac. erythraea* was genetically modified to be deficient in the erythromycin C-6 and C-12 hydroxylases. This was accomplished by first replacing the wild type copy of *eryK* (encoding the C-12 hydroxylase) with a deleted copy using plasmid pDPE4, described in Example 1. As predicted from the
20 proposed pathway for erythromycin biosynthesis, the mutant strain produced erythromycin B, with some erythromycin D also being produced early in the fermentation. The *eryF* gene (encoding the C-6 hydroxylase) of this mutant strain was then replaced by a deleted copy of the gene using plasmid pGM504, described in Example 2. The expected product of this doubly deleted strain, 6,12-dideoxyerythromycin A, was made but the strain also produced
25 large amounts of 6-deoxyerythromycin D throughout a six day fermentation, with the 6-deoxyerythromycin D being the dominant derivative from days 1 to 6, as shown in FIG. 6.

 In order to produce highly pure 6,12-dideoxyerythromycin A, an extra copy of *eryG* was introduced into a non-essential region of the *Sac. erythraea* chromosome. The product of *eryG* is the 3"-O-methyltransferase which normally converts erythromycins D and C to
30 erythromycin B and A, respectively. The preferred method for constructing a gene replacement plasmid for the addition of a second copy of *eryG* into the *Sac. erythraea* chromosome is shown schematically in FIG. 4 and described in Example 3. In a preferred embodiment of the present invention, a selectable DNA plasmid is constructed which comprises (a) a fragment of plasmid pCD1 containing an origin of replication functional in
35 *Streptomyces* and *Saccharopolyspora*; (b) an origin of replication and a DNA fragment conferring resistance to the antibiotic ampicillin, each of which are functional in *E. coli*; and (c) a DNA fragment from a region of the *Sac. erythraea* chromosome of unknown, but non-essential function capable of acting as a recognition site for plasmid integration and excision.

- 7 -

(Hereafter, this DNA fragment of unknown but non-essential function is referred to as the 'second site' region. FIG. 13 depicts approximately 1 kb of single stranded DNA sequence which is a portion of the second site region). Embedded within the 'second site' region are two additional DNA fragments, one encoding 3"-O-methyltransferase operably linked to the *ermE** promoter, and a second fragment from plasmid pWHM3 (also referred to herein as pCS5) which confers resistance to the antibiotic thiostrepton. A culture of *E. coli* DH5 α which contains a plasmid embodying the invention, designated pDPE35, has been deposited with the Agricultural Research Culture Collection, Peoria, Illinois and has been accorded the accession number NRRL B-21486.

As in the previous Examples, the particular antibiotic resistance genes and functional origins of replication identified above are necessary only inasmuch as they allow for the selection and replication of the desired recombinant plasmid. Other markers and origins of replication may be used. Likewise, any DNA fragment which is homologous to a non-essential region of the *Sac. erythraea* chromosome may be used as the integration/excision recognition sequences surrounding the *eryG* and *tsr* genes. Example 5 describes the use of pDPE35 in the construction of a strain of *Sac. erythraea*, which had previously been deleted in *eryK* and *eryF*, and which now contains an extra copy of *eryG*. This extra copy of *eryG* allows the production of high purity 6,12-dideoxyerythromycin A over at least a 4 day fermentation period, as shown in Fig 7. The *Sac. erythraea* strain having deletions in *eryK* and *eryF*, referred to herein as strain ER720-KFG+, has been deposited with the Agricultural Research Culture Collection, Peoria, Illinois and has been accorded accession number NRRL 21484.

A. DEFINITIONS

The following words and phrases have the meaning set forth below.

The term "cytochrome P-450 monooxygenase system" as used herein refers to a group of proteins (two flavoproteins, an iron-sulphur protein and the C-6 or C-12 hydroxylase enzymes) which function together to cause hydroxylation of erythromycin B or its derivatives in *Sac. erythraea*. The term "cytochrome P-450 enzymes" refers to the C-6 or C-12 hydroxylase enzymes of the cytochrome P-450 monooxygenase system.

The term "erythromycin derivative" as used herein refers to any erythromycin-like compound having antibiotic and/or prokinetic activity. Erythromycin-like compounds are typically characterized by having a 14-membered macrolactone ring and two O-linked sugar molecules, such as are found in erythromycins A, B, C and D. "Erythromycin derivatives" are intended to include erythromycin-like compounds having modifications and/or substituents in the macrolactone ring and/or sugar portions, provided they serve as substrate for 3"-O-methyl transferase. For example, common known modifications include:

4" deoxyerythromycin;

- 8 -

6-deoxyerythromycin D;
 6,9 epoxyerythromycin;
 6-O-methylethromycin;
 4"-amino-6,4"-dideoxyerythromycin A;
 5 9,4"-diamino-6,9,4"-trideoxyerythromycin A;
 8,9-anhydro-4"-deoxyerythromycin A-6,9-hemiketal;
 8,9-anhydro-4"-deoxyerythromycin B-6,9-hemiketal;
 8,9-anhydro-4"-deoxy-3'-N-desmethylethromycin A-6,9-hemiketal;
 8,9-anhydro-4"-deoxy-3'-N-desmethyl-3'-N-ethylethromycin A-6,9-hemiketal;
 10 8,9-anhydro-4"-deoxy-3'-N-propargylethromycin A-6,9-hemiketal bromide;
 8,9-anhydro-4"-deoxy-3'-N-desmethylethromycin B-6,9-hemiketal;
 8,9-anhydro-4"-deoxy-3'-N-desmethyl-3'-N-ethylethromycin B-6,9-hemiketal;
 8,9-anhydro-4"-deoxy-3'-N-propargylethromycin B-6,9-hemiketal bromide;
 9-deoxo-4",6-dideoxy-8-epi-6,9-epoxyerythromycin A;
 15 9-deoxo-3'-N-desmethyl-4",6-dideoxy-8-epi-6,9-epoxyerythromycin A;
 9-deoxo-3'-N-desmethyl-4",6-dideoxy-8-epi-3'-N-ethyl-6,9-epoxyerythromycin A;
 9-deoxo-4",6-dideoxy-8-epi-6,9-epoxy-3'-N-propargylethromycin A bromide;
 9-deoxo-4",6-dideoxy-6,9-epoxyerythromycin A;
 9-deoxo-3'-N-desmethyl-4",6-dideoxy-6,9-epoxyerythromycin A;
 20 9-deoxo-3'-N-desmethyl-4",6-dideoxy-6,9-epoxy-3'-N-ethylethromycin A; and
 9-deoxo-4",6-dideoxy-6,9-epoxy-3'-N-propargylethromycin A bromide.

The term "expression" as used herein refers to the combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

25 The term "homologous recombination" as used herein refers to complementary base-pairing and crossing over between DNA strands containing identical or nearly identical sequences.

The terms "origin of replication" as used herein refers to a DNA sequence that controls and allows for replication and maintenance of a plasmid or other vector in a host cell.

30 The term "operably linked" as used herein refers to the control exerted by the promoter over the initiation of transcription of a structural gene.

The term "promoter" as used herein refers to a recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of
 35 that gene.

The term "restriction fragment" as used herein refers to any linear DNA generated by the action of one or more restriction enzymes.

The term structural gene refers to a gene that is expressed to produce a polypeptide.

- 9 -

The term "transformation" as used herein refers to a process of introducing an exogenous DNA sequence (e.g. a vector, a recombinant DNA molecule) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

5 The term "vector" as used herein refers a DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

B. BACTERIAL STRAINS, PLASMID VECTORS, AND GROWTH MEDIA

10 The erythromycin-producing microorganism used to practice the following examples of the invention was *Sac. erythraea* ER720 (DeWitt, J.P. *J. Bacteriol.* 164: 969 (1985)). The host strain for the growth of *E. coli* derived plasmids was DH5 α from Bethesda Research Laboratories (BRL), Gaithersburg, MD.

15 Plasmid pUC18, pUC19 and pBR322 can be obtained from BRL. Plasmid pCS5, is a multifunctional vector for integrative transformation of *Sac. erythraea*. (Plasmid pCS5 has been described by Vara, et al. *J. Bacteriol.* 171(11): 5872 (1989) and was originally designated as pWHM3). Plasmids pIJ702 (described by Katz, et al. *J. Gen. Microbiol.* 129: 2703 (1983)) and pIJ4070 were obtained from the John Innes Institute. Plasmid pCD1 was obtained from Claude Dery, University of Sherbrook, Quebec, Canada. Restriction map
20 analysis and partial sequencing have shown this plasmid to be related to pJV1 described by Doull, J.L. et al. *FEMS Microbiol. Lett.* 16: 349 (1983).

Sac. erythraea was grown for protoplast transformation and routine liquid culture in 50 ml of SGGP medium (Yamamoto, et al., *J. Antibiot.* 39:1304 (1986)), supplemented with 10 micrograms/milliliter (μ g/mL) of thiostrepton for plasmid selection where appropriate.

C. REAGENTS AND GENERAL METHODS

25 Commercially available reagents were used to make compounds, plasmids and genetic variants of the present invention, including ampicillin, thiostrepton, (purchased from Sigma Chemical Co., St .Loius, MO) restriction endonucleases, T4-DNA ligase, and calf intestine alkaline phosphatase (CIAP) (purchased from New England Biolabs, Beverly, MA).

Standard molecular biology procedures (Maniatis, et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory (1982)) were used for the construction and characterization of integrative plasmids. Plasmid DNA was routinely isolated by the alkaline lysis method (Birnboim, H.C. and Doly, J., *Nucleic Acids Res.* 7: 1513 (1979)).

35 Restriction fragments were recovered from 0.8-1% agarose gels with either Prep-A-Gene (BioRad, Hercules, CA) or Gene Clean II (Bio101, Vista CA). The products of ligation for each step of plasmid constructions were used to transform the intermediate host, *E. coli* DH5 α (purchased from BRL), which was cultured in the presence of ampicillin to select for host

- 10 -

cells carrying a recombinant plasmid. Screening for the presence of insert DNA with X-gal was used where appropriate. Plasmid DNAs were isolated from individual transformants that had been grown in liquid culture and were characterized with respect to known restriction sites.

Integrative transformation of *Sac. erythraea* protoplasts, and routine growth and sporulation were carried out according to procedures described in Donadio, et al., *Science* 115: 97 (1991), Weber and Losick, *Gene* 68: 173 (1988) and Yamamoto, et al., *J. Antibiot.* 39:1304 (1986).

The following abbreviations are used throughout the application:

- a. TES: N-tris(Hydroxymethyl)methyl-2-aminoethane-sulfonic acid
- b. R3M: A growth medium containing per 1 liter aqueous solution: 103 g sucrose, 0.25 g K₂SO₄, 4 g yeast extract, 4 g casamino acids, 4 g tryptone, 22 grams agar in 830 mL of H₂O. The solution is sterilized by autoclaving. After sterilization, the following additional ingredients are added: 20 mL of 2.5 M MgCl₂, 20 mL of 50% glucose, 20 mL of 2.5 M CaCl₂, 12.5 mL of 2 M Tris-HCl, pH 7.0, 2 mL of trace elements solution (Hopwood, et al, 1985, Genetic Manipulation of Streptomyces A Laboratory Manual, The John Innes Institute), 0.37 mL of 0.5 M KH₂PO₄ and 2.5 mL of NaOH.
- c. P_M: A buffer containing per 1 liter aqueous solution: 200 grams (g) sucrose, 0.25 g K₂SO₄ in 890 mL H₂O, with the addition after sterilization of 100 mL 0.25 M TES, pH7.2, 2 mL trace elements solution (Hopwood, et al, 1985, Genetic Manipulation of Streptomyces A Laboratory Manual, The John Innes Foundation), 0.08 mL 2.5 M CaCl₂, 10 mL 0.5% KH₂PO₄, 2 ml 2.5M MgCl₂.
- d. A4Bf: A growth medium containing per 1 liter aqueous medium: 15 g soy flour, 50 g glucose, 5 g NaCl, and 1 g CaCO₃.
- e. SCM: A growth medium containing per 1 liter aqueous medium: 20 g soytone, 15 g soluble starch, 10.5 g MOPS, 1.5 g yeast extract and 0.1 g CaCl₂.

The foregoing can be better understood by reference to the following examples, which are provided as non-limiting illustrations of the practice of the instant invention. Both below and throughout the specification, it is intended that citations to the literature be expressly incorporated by reference.

EXAMPLE 1

Construction of plasmid pDPE4

pDEP4 was constructed using standard methods of recombinant DNA technology according to the schematic outline shown in FIG. 2. A 2.55 kb *EcoRI* -*PstI* fragment containing *eryK* and flanking portions of ORF 19 and ORF 21 was isolated from pEVEH8 and ligated to pUC18 cut with the same enzymes to generate plasmid

- 11 -

pDPE1. This plasmid was then cut with *Eco*0109I and two of the three fragments generated (i.e. those having sizes of 0.9 and 4.3 kb) were isolated. These two fragments were ligated to generate pDPE2, which contains a small deletion within the *eryK* gene. A 2.1 kb *Eco*RI-*Pst*I fragment from pDPE2 was then ligated to pCS5 cut with the same enzymes to yield pDPE3. Additional contiguous DNA sequence was added downstream of *eryK* by excising a 0.765 kb *Pst*I fragment containing ORF 19 from pEVEH8 and ligating this to *Pst*I cut, CIAP treated pDPE3, to generate pDPE4. Orientation was confirmed by restriction analysis.

EXAMPLE 2

Construction of plasmid pGM504

pGM504 was constructed using standard methods of recombinant DNA technology according to the schematic outline shown in FIG. 3. pGM420, a *Streptomyces-E. coli* shuttle vector, was constructed by cutting pUC18 with *Sst*I and ligating this plasmid into the *Sst*I site of pIJ702. The pUC18 polylinker is oriented proximal to the *Bgl*II, *Sph*I and *Asp*718 sites of pIJ702. A 5.3kb *Pst*I fragment of *Sac. erythraea* DNA containing *eryF*, flanking and nearby DNA including part of *eryG* was cloned into the *Pst*I site of pGM420 to give pMW65. A 0.5 kb out of frame deletion in *eryF* was made by sequential partial digestions of pMW65 with *Asp*718 and *Sst*I, and then filling in the sticky ends with pol1K and religating to yield pGM504.

EXAMPLE 3

Construction of plasmid pDEP35

pDPE35 was constructed using standard methods of recombinant DNA technology according to the schematic outline shown in FIG. 4. A 4.5 kb *Eco*RI-*Bam*HI fragment from cosmid p7A2 (Paulus et al., *J. Bacteriol.* 172: 2541 (1990)) containing *eryG* was ligated to pBR322 cut with the same enzymes, to give pGM403. The *Eco*RI-*Sph*I fragment of pGM403 containing *eryG* was then ligated to pUC18 cut with the same enzymes to generate pDPE8. The *ermE** promoter (carried on an *Eco*RI-*Bam*HI fragment from pIJ4070) was inserted upstream of *eryG* into the *Eco*RI-*Bgl*II sites of pDPE8 to create pKAS2. pKAS2 was digested to completion with *Eco*RI and then partially with *Nae*I in order to isolate the 1.5 kb fragment containing the *ermE**-*eryG* fusion. This fragment was ligated to pUC18 cut with *Eco*RI and *Hinc*II to generate pKAS3. pKAS3 was digested with *Ssp*I and *Sph*I to obtain a 3.4 kb fragment; this fragment was ligated to the 0.6 kb fragment of pUC19 cut with the same enzymes in order to add an *Eco*RI site downstream of *eryG* to generate pKAS4.

The *eryG* gene was inserted into the *Sac. erythraea* DNA 'second site' region in the following manner. An 11 kb *Hind*III fragment of *Sac erythraea* chromosomal DNA was

- 12 -

ligated to a pBR322 derivative to generate pGM469. This *Hind*III fragment contains a unique *Stu*I site into which was inserted an *Eco*RI-*Stu*I linker, to generate pGM473. This plasmid was digested with *Eco*RI and treated with CIAP. The 1.6 kb *Eco*RI fragment from pKAS4 containing the *ermE** - *eryG* fusion was isolated and ligated to pGM473 to generate pKAS19. The 14 kb *Hind*III fragment of pKAS19 containing the 'second site' region construct was then ligated to pCD1 cut with *Hind*III and treated with CIAP to yield pKAS20.

The thiostrepton resistance gene was placed downstream of *eryG* in the following manner. A 1.1 kb *Bcl*II fragment containing the *tsr* gene from plasmid pCS5 was inserted into pUC19 (cut with *Bam*HI and treated with CIAP) to generate pDPE23A. In order to insert a multiple cloning site (MCS) downstream of *tsr*, this plasmid was digested with *Eco*RI and *Sca*I and ligated to pUC18 cut with the same enzymes to give pDPE26. A 1.1 kb *Xba*I fragment containing *tsr* could then be isolated from pDPE26 and ligated to *Xba*I cut and CIAP treated pKAS20 to generate pDPE34.

Removal of the second copy of *tsr* from pDPE34 was accomplished in the following manner. The 3 kb *Nde*I-*Eco*RI fragment from pCD1 containing a *Sac. erythraea* origin of replication was ligated to pUC19 digested with the same enzymes to give plasmid pDPE21. The 15 kb *Hind*III fragment of *Sac. erythraea* DNA containing the *ermE** promoter, *eryG* and *tsr* from pDPE34 was then ligated into the *Hind*III site of pDPE21 to give plasmid pDPE35.

EXAMPLE 4

Construction of *Sac. erythraea eryK, eryF* strain (ER720-KF)

An example of a 6,12-dideoxyerythromycin A producing microorganism was prepared by replacing the wild type *eryK* and *eryF* of *Sac. erythraea* ER720 cells with deletions in these genes carried on the recombinant plasmids of Examples 1 and 2. Transformation and resolution of the integration event was carried out according to the following method. *Sac. erythraea* ER720 cells were grown in 50 mL of SGGP medium for 3 days, at 32 °C and then washed in 10 mL of 10.3% sucrose. The cells were resuspended in 10 mL of P_M buffer containing 1 mg/mL lysozyme and incubated at 30 °C for 15-30 minutes until most of the mycelial fragments were converted into spherical protoplasts. The protoplasts were washed once with P_M and then resuspended in 3 mL of the same buffer containing 10% DMSO for storage in 200 mL aliquots at -80 °C.

Transformation was carried out by quickly thawing an aliquot of protoplasts, centrifuging for 15 seconds in a microfuge, decanting the supernatant, and resuspending the protoplasts in the P_M remaining in the tube. Ten µL of DNA solution was added (3 µL of pDPE4 DNA from Example 1 at about 1 µg/µL in 7 µL of P_M buffer) and mixed with the protoplasts by gently tapping the tube. Two tenths of a mL of 25% PEG 8000 in T buffer (Hopwood, et al, 1985, Genetic Manipulation of Streptomyces A Laboratory Manual, The

- 13 -

John Innes Institute) was then added, mixed by pipetting the solution three times and the suspension immediately spread on a dried R3M plate. The plate was incubated at 30 °C for 20 hours and overlaid with 2 mL of water containing 100 µg/mL thiostrepton, dried briefly and incubated 4 more days at 30 °C.

To select integrants, transformants were replica plated onto non-selective R3M medium (i.e. without thiostrepton), allowed to sporulate and then replica plated onto R3M medium containing 10 µg/mL thiostrepton. 10 colonies were inoculated into SGGP containing thiostrepton. Of these, 8 grew and were selected as integrants. Integration of the plasmid DNA was confirmed by Southern hybridization, and all 8 strains were found by TLC analysis to make erythromycin A.

The 8 integrants were then grown non-selectively on R3M and allowed to sporulate. Spores were plated to obtain individual colonies on R3M plates, which were then screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Eight thiostrepton sensitive colonies were selected and two of these were confirmed by Southern hybridization and by the production of erythromycins D and B to contain the deleted copy of *eryK* in the chromosome.

Replacement of *eryF* with a deleted copy was performed as described above for the *eryK* deletion, except that the *eryK* deleted strain was used as the recipient of pGM504 (described in Example 2). Integration and excision of the plasmid from the *Sac. erythraea* chromosome was monitored by Southern analysis, and the resulting strain, named ER720-KF, was found to produce a mixture of 6,12-dideoxyerythromycin A and 6-deoxyerythromycin D.

EXAMPLE 5

Construction of *Sac. erythraea eryK*, *eryF*,
'second site' ::*eryG*+ (ER720-KFG+)

A preferred example of the 6,12-dideoxyerythromycin A producing microorganism of the present invention was prepared by transforming ER720-KF cells with the recombinant plasmid of Example 3 (i.e. pDPE35) to construct a strain which produces highly pure 6,12-dideoxyerythromycin A rather than a mixture of 6,12-dideoxyerythromycin A and 6-deoxyerythromycin D. Integration and excision of pDPE35 from the *Sac. erythraea* chromosome to leave behind a second copy of *eryG* driven by the *ermE** promoter was performed as follows. Protoplasts of ER720-KF cells were transformed with pDPE35 as described in Example 4. In order to resolve the duplication created by the integration of the plasmid at a region of homology of unknown but non-essential function in the *Sac. erythraea* chromosome, and as a result leave behind the *eryG* and the thiostrepton resistance marker carried by that plasmid, transformants were streaked two consecutive times on R3M plates containing thiostrepton. Those colonies which were able to grow after two passages on

- 14 -

thiostrepton were found by Southern analysis to contain a second copy of *eryG* integrated into the 'second site' region of the chromosome. The strain was designated ER720-KFG+.

EXAMPLE 6

5 Fermentation of ER720-KF and ER720KFG+, and Identification of Compounds Produced by the 2 Strains

10 The recombinant *Sac. erythraea* strains produced in Examples 4 and 5 were cultivated using the following fermentation procedure. Six hundred mL seed cultures of ER720-KF and ER720KFG+ were grown in A4Bf medium in cotton plugged 2-liter flasks at 32°C, at 225 rpm for 48 and 72 hours, respectively. Forty-five liter LH fermenters (Incel Tech, Hayward, CA) containing 30 liters of SCM medium (with thiostrepton added to 10 µg/mL for ER720-KFG+) were inoculated with 1.5 liters of seed culture. Cells were grown at 32°C, at 250 rpm with a head pressure at 5 psi, and an aeration rate of 0.7-1 volumes of O₂/volume of
15 culture/minute. Antifoam was added to 0.01% initially and pH was controlled at 7.0 with propionic acid and KOH. Culture samples were taken at 0, 24, 40, 48, 66, 72 and 144 hours for ER720-KF and 24, 40, 48, 66, 72, 88 and 144 hours for ER720-KFG+.

20 Erythromycin derivatives were isolated from the culture broth of the producing strains by the following procedure. Cells were removed from 1.5 mL of culture by centrifugation for one minute in a microfuge. One mL of the supernatant was removed to another tube and the pH adjusted to 9.0 by the addition of 6 µL NH₄OH. One half mL of ethyl acetate was added, the tube was vortexed for 10 sec and then centrifuged for approximately 5 minutes to separate the phases. The organic phase was removed to another tube, and the aqueous phase was re-extracted with 0.5 mL of ethyl acetate. The second organic phase was pooled with the first
25 and dried in a Speed Vac. The residue was taken up in 11 µL of ethyl acetate and 1 µL was spotted onto TLC plates. A standard curve of 6,12-dideoxyerythromycin A was also included to insure that the amounts of compound applied to the plate were in the linear range of the detection method.

30 Silica gel thin-layer chromatography plates (Merck 60F-254) were developed using isopropyl ether-methanol-NH₄OH (75:35:2). Compounds were visualized by spraying the plates with anisaldehyde-sulfuric acid-ethanol (1:1:9). With this reagent, 6,12-dideoxyerythromycin A and 6-deoxyerythromycin D appear as blue spots and are additionally identified by comparing their R_f values (ratio of movement of the spot to the movement of the solvent front) with that of standards (see Figs. 6 and 7).

35 The ratio of 6,12-dideoxyerythromycin A to 6-deoxyerythromycin D produced by the genetically engineered strains was analyzed by measuring TLC spots with a Molecular Dynamics Personal Densitometer (PD-120 laser based transmission scanner) at 100 µm resolution. FIG. 8a demonstrates that over a 6 day fermentation, while the strain lacking the

- 15 -

C-6 and C-12 hydroxylases produced 6,12-dideoxyerythromycin A, it also accumulated a large amount of the non-methylated precursor, 6-deoxyerythromycin D. However, as shown in FIG. 8b, when an extra copy of the 3"-O-methyltransferase gene was added to a non-essential region of this strain, it was able to overcome the accumulation of 6-deoxyerythromycin D, and convert this precursor to highly pure 6,12-dideoxyerythromycin A.

EXAMPLE 7

Construction of plasmid pKAS37

pKAS37 was constructed using standard methods of recombinant DNA technology according to the schematic outline in FIG. 9. The *ermE** promoter from pIJ4070 was inserted into the *Bam*HI/*Eco*RI sites of pUC19, to give pKAS7. A region of the polylinker including the *Kpn*I to *Bgl*II sites was moved from pIJ4070 to pUC19 to give pKAS8. pKAS7 was digested with *Ssp*I/*Bam*HI and the *ermE** promoter fragment inserted into *Ssp*I/*Bgl*II digested pKAS8 to give pKAS16. To eliminate a *Pvu*II site, plasmid pKAS16 was digested with *Nae*I/*Eco*O109, filled-in with Klenow and ligated to generate pKAS17. pKAS17 was then digested with *Ssp*I/*Hind*III and the *ermE** fragment ligated into a similarly digested pIJ4070 to give pKAS18.

The *tsr* gene was excised from pCS5 by *Bcl*I digestion and ligated into *Bam* HI digested pUC19 dephosphorylated with CIAP to give pDPE23B. pDPE23B was then digested with *Ssp*I/*Nde*I and the *ermE** fragment was isolated from pKAS18 digested with *Ssp*I/*Ase*I and ligated to generate pKAS23. pKAS23 was digested with *Sst*II/*Pvu*II and the 1.1 kb *Sst*II/*Sma*I fragment from pCS5 were ligated to give pKAS33.

pDPE35 was digested with *Hind*III/*Kpn*I and the pCD1 replicon fragment ligated to a similarly digested pGM469 to give pKAS30. pKAS30 was digested separately with *Hind*III and *Kpn*I with concurrent fill-in with Klenow to give pKAS34. pKAS34 was partially digested with *Stu*I, dephosphorylated with CIAP and the fragment containing *ermE** and *tsr* gene from pKAS33 digested with *Ssp*I/*Fsp*I were ligated to generate pKAS35(-). pDPE36 was generated by digesting pDPE21 with *Mlu*I/*Nde*I, filling-in with Klenow and ligating. pKAS35(-) was digested with *Eco*RI/*Stu*I and the pCD1 replicon from pDPE36 similarly digested were ligated to generate pKAS36. pKAS36 and pKAS35(-) were digested with *Ssp*I/*Eco*RI to generate pKAS37. A detailed restriction map of this plasmid is shown in FIG. 10. A culture of *E. coli* DH5 α which contains plasmid pKAS37 has been deposited as above with the Agricultural Research Culture Collection and has been accorded the accession number NRRL B-21485.

- 16 -

EXAMPLE 8

Construction of plasmid pKASI37

pKASI37, an alternative embodiment of pKAS37, is constructed using standard methods of recombinant DNA technology according to the schematic outline in Fig 11. To obtain the 'second site' region of pKASI37, *Sac. erythraea* ER720 or NRRL2338 chromosomal DNA is digested with *Hind*III and fragments of approximately 12 kb are isolated from a 0.7% agarose gel. This pool of fragments is ligated to pUC19 (also digested with *Hind*III). Transformants are screened for plasmids carrying the second site by digestion of miniprep DNA with *Bam*HI, *Mlu*I and *Stu*I to generate expected fragments of 6.8, 5.6 and 2.1 kb (for *Bam*HI), 11.3, 3.3 and 0.3 kb (for *Mlu*I) and 14.9 kb (for *Stu*I). Proper orientation of the *Hind*III fragment is determined by *Kpn*I digestion as the *Kpn*I site at one end of the 'second site' region should be adjacent to the *Kpn*I site in the pUC19 polylinker. The resulting plasmid is pX1.

Plasmid pCD1 is then digested with *Mlu*I, treated with Klenow and digested with *Kpn*I. The resulting fragment of about 3 kb containing the *Sac. erythraea* replicon is ligated to pX1 digested with *Nde*I (filled in with Klenow) and *Kpn*I to form plasmid pX2. Plasmid pX2 is then digested with *Kpn*I, treated with Klenow and religated to give plasmid pX3. pX3 is digested with *Hind*III, treated with Klenow and religated to give plasmid pX4.

The final construction steps of the plasmid involve insertion of the *ermE** promoter, polylinker and *tsr* gene. Plasmid pIJ4070 is digested with *Kpn*I, treated with Klenow and religated to form plasmid pX5. Two oligonucleotides are then synthesized which when annealed will contain the following restriction sites: *Bgl*II-*Eco*RI-*Kpn*I-*Xba*I-*Hind*III-*Bgl*II-*Bam*HI-*Eco*RI-*Pst*I (synthesized polylinker). This double stranded fragment is ligated into pX5 digested with *Bam*HI and *Pst*I to give plasmid pX6. Plasmid pCS5 is then digested with *Bcl*II and the resulting 1 kb *tsr* containing fragment is ligated into the *Bam*HI site of pX6 to give plasmid pX7. pX7 is then partially digested with *Eco*RI, treated with Klenow and the 1.4 kb DNA fragment containing the *ermE** promoter-synthesized polylinker-*tsr* gene is inserted into the unique *Stu*I site of the *Sac. erythraea* 'second site' region in pX4 to form plasmid pKASI37. A detailed restriction map of plasmid pKASI37 is shown in FIG. 12.

- 17 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Diane L. Stassi
Gregory T. Maine
David A. Post
Mark Satter

(ii) TITLE OF INVENTION: VECTORS AND PROCESS FOR PRODUCING HIGH PURITY 6,12-DIDEOXYERYTHROMYCIN A BY FERMENTATION

(iii) NUMBER OF SEQUENCES:1

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Abbott Laboratories
(B) STREET: 100 Abbott Park Road
(C) CITY: Abbott Park
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60064-3500

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patentin Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Andreas M. Danckers
(B) REGISTRATION NUMBER: 32,652
(C) DOCKET NUMBER: 5789.US.01

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 708/937-9803
(B) TELEFAX: 708/938-2623
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 917 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: genomic DNA
(iii) HYPROTHETICAL: No

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE: *Saccharopolyspora erythraea*

(ix) FEATURE:

(A) NAME/KEY: 1kb portion of second site region
(B) LOCATION:
(C) OTHER INFORMATION: Non-essential function

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

- 18 -

GAGCGACCAC AGGTGGGCCC GGATGTTGCA GCCTTGGTCTG GGGTAGTCGA TGCGGATTCTG	60
GAACAGTGCC ACGGCTGTGG TGTTCGAAGG TGGAAGTCTT GAGCTGCTGG TGCCACCGGA	120
TTGCTTGCTC CAGCGAGACC GCGTTGCCGT TGACGAAGGC CAACGCGTCA AACACCGCCT	180
GGGAGTGCTC GGGTCGCAGT TTCTTCAAGT CATCGCTGAG AATCCCGGCA CCGAGCGTGA	240
TAGGCATCCT GCACCGCCCC ACACGGCGCG GAGATTGCGG TCCAGGCCCG GCAACATACC	300
AGCGCTTCGT CGAACTCGTC CGCCTCGACG TGGGCCCCGA GTTGTTCCGC GAACACTGCG	360
CAGTTCGGAG CAGCTTCTGG CCCAGGGCTT GCGACAACCT TGGGTGGGGT GTGCGCGGGG	420
TTGGTGCTGA AGTCGTTGCG GAAACCCAGC ATCGTCAGAG CGTGGTCGAA CTGTGCTGGA	480
CTGAGGTGCT CAGACAGCAC ACGAATCCAG CTCCCTGCCG GTGTGCTGCC AGAAGGGGAC	540
CGCGAGGCCC GCGGAATCTC CGCCGGATCG CCCCGAAGCC GACCCAGCTC ACGCAACACC	600
GAATCGGTGT CCGGCCGAGG TGACCGTGTG CCCGACCCGG AGCCGGGAGC ACGCCGCGCA	660
CTGGGCCTCC TCGGTTGTGT GTGTGAGATC GTCGTTCCCTC GAATTTAAGC AAGCCGGCGA	720
TGAACTTCGC CCGGCGCGCG GACAACGTCG TCACATCACC GTCCGCCCCG ACGCCAGAAG	780
CCGAGCCAGC CCCC GCACTG CGCCCCGAAC GGAACCTCCT CGGAAGTTAC GCCGGAGCTG	840
CCCGGTGCCG CCGTGGTCAG GAAAGCCTGC GCGTGCTGAG GGAGCCGTCC ATGTTGATAA	900
TTATTATCTC AGATGAC	917

We claim:

1. A recombinant DNA vector for integrating a DNA sequence of interest into the chromosome of an erythromycin producing host cell, said vector comprising a first DNA sequence of a non-essential region of the *Saccharopolyspora erythraea* chromosome, a second DNA sequence which allows for replication in a particular host cell and a third DNA sequence encoding a selectable marker gene.
2. The DNA vector of claim 1 wherein said second DNA sequence is derived from plasmid pCD1.
3. The DNA vector of claim 1 further comprising a DNA sequence which encodes the *ermE** promoter wherein said *ermE** promoter is operably linked to a gene of interest.
4. The DNA vector of claim 3 wherein said gene of interest is the *eryG* gene.
5. The DNA vector of claim 1 further comprising a multiple cloning site.
6. The DNA vector of claim 1 wherein said vector is plasmid pKAS37.
7. The DNA vector of claim 6 further comprising the *eryG* gene.
8. The DNA vector of claim 1 wherein said vector is plasmid pDPE35.
9. A method for making a substantially pure 3''-methylated erythromycin derivative, said method comprising:
 - a. introducing an integrative recombinant vector containing the *eryG* gene into host cells wherein said host cells produce a mixture of said 3''-methylated erythromycin derivative and an 3''-unmethylated erythromycin derivative;
 - b. selecting for stable integrants of said host cells, said integrants having said *eryG* gene stably integrated into a non-essential region of the chromosome of said integrants;
 - c. culturing said stable integrants in a culture medium; and
 - d. isolating said 3''-methylated erythromycin derivative from said culture medium.
10. The method of claim 9 wherein said integrative recombinant DNA vector is the vector of claim 1, claim 4, claim 6 or claim 8.

- 20 -

11. A method for making substantially pure 6,12-dideoxyerythromycin A, said method comprising:

a. introducing an integrative recombinant vector containing the *eryG* gene in host cells which produce a mixture of said 6,12-dideoxyerythromycin A and 6-deoxyerythromycin D;

b. selecting for stable integrants of said host cells; said integrants having said *eryG* gene stably integrated into a non-essential region of the chromosome of said integrants;

c. culturing said stable integrants in a culture medium; and

d. isolating said 6,12-dideoxyerythromycin A from said culture medium.

12. The method of claim 14 wherein said integrative recombinant DNA vector is the vector of claim 1, claim 4, claim 6 or claim 8.

13. A method for increasing 3''-O-methyltransferase activity in host cells which produce at least one substrate for said 3''-O-methyltransferase activity, said method comprising:

a. introducing an integrative recombinant vector containing said *eryG* gene into said host cells, said *eryG* gene encoding said 3''-O-methyltransferase activity; and

b. selecting for stable integrants of said host cells, said integrants having said *eryG* gene stably integrated into a non-essential region of the chromosome of said integrants.

14. The method of claim 19 wherein said integrative recombinant DNA vector is the vector of claim 1, claim 4, claim 6 or claim 8.

15. A method for making a modified deoxyerythromycin producing host strain, wherein said modification is an increase in 3''-O-methyltransferase activity, said method comprising:

a. introducing an integrative recombinant vector containing the *eryG* gene into said host strain, said *eryG* gene encoding said 3''-O-methyltransferase activity; and

b. selecting stable integrants of said host strain, said integrants having said *eryG* gene stably integrated into a non-essential region of the chromosome of said integrants.

16. The method of claim 24 wherein said integrative recombinant DNA vector is the vector of claim 1, claim 4, claim 6 or claim 8.

17. A host strain which produces an erythromycin derivative, said host strain being modified to overproduce 3''-O-methyltransferase activity wherein said modification is stable integration the *eryG* gene encoding said 3''-O-methyltransferase activity into a non-essential region of the chromosome of said host strain.

1/18

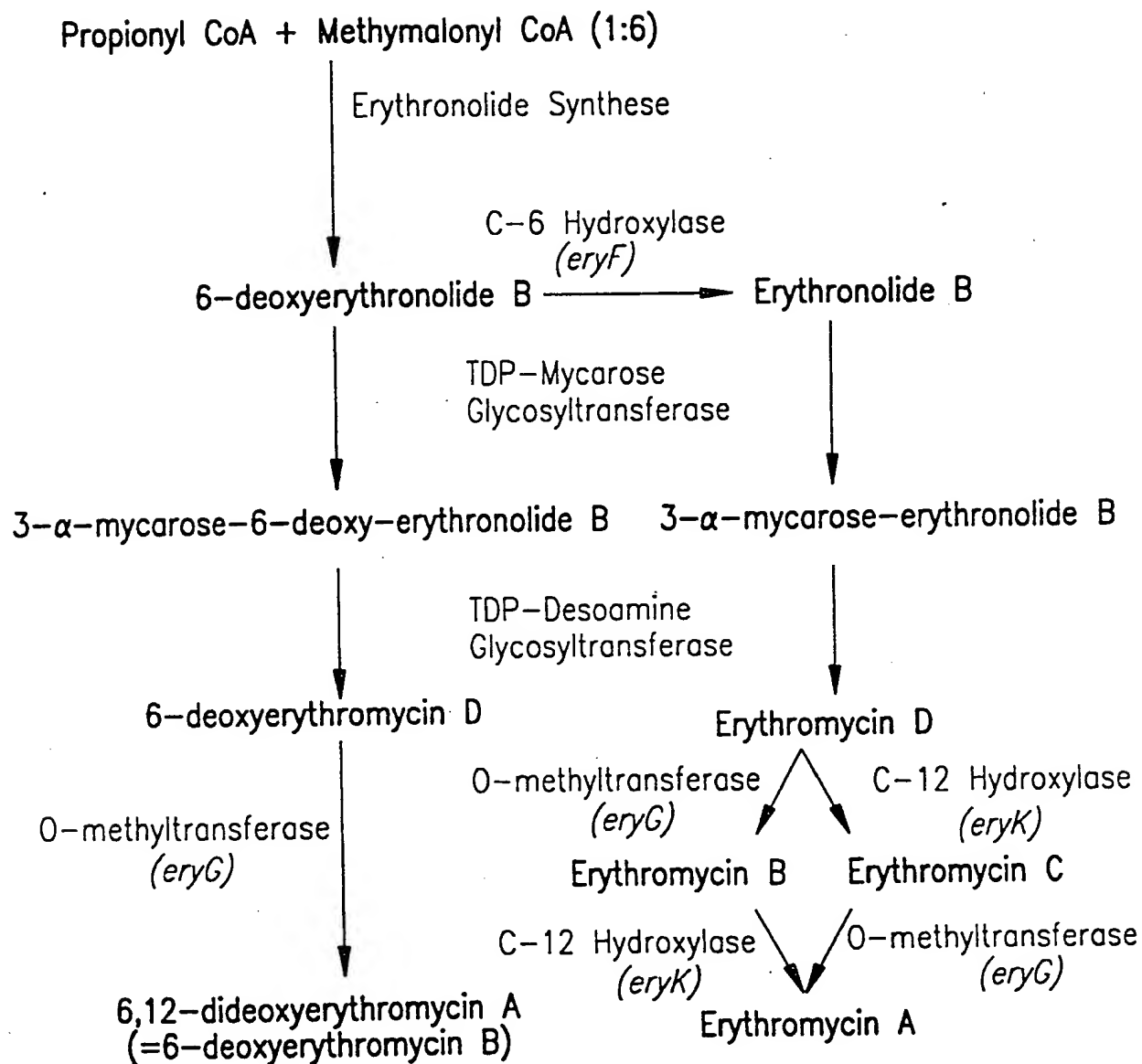
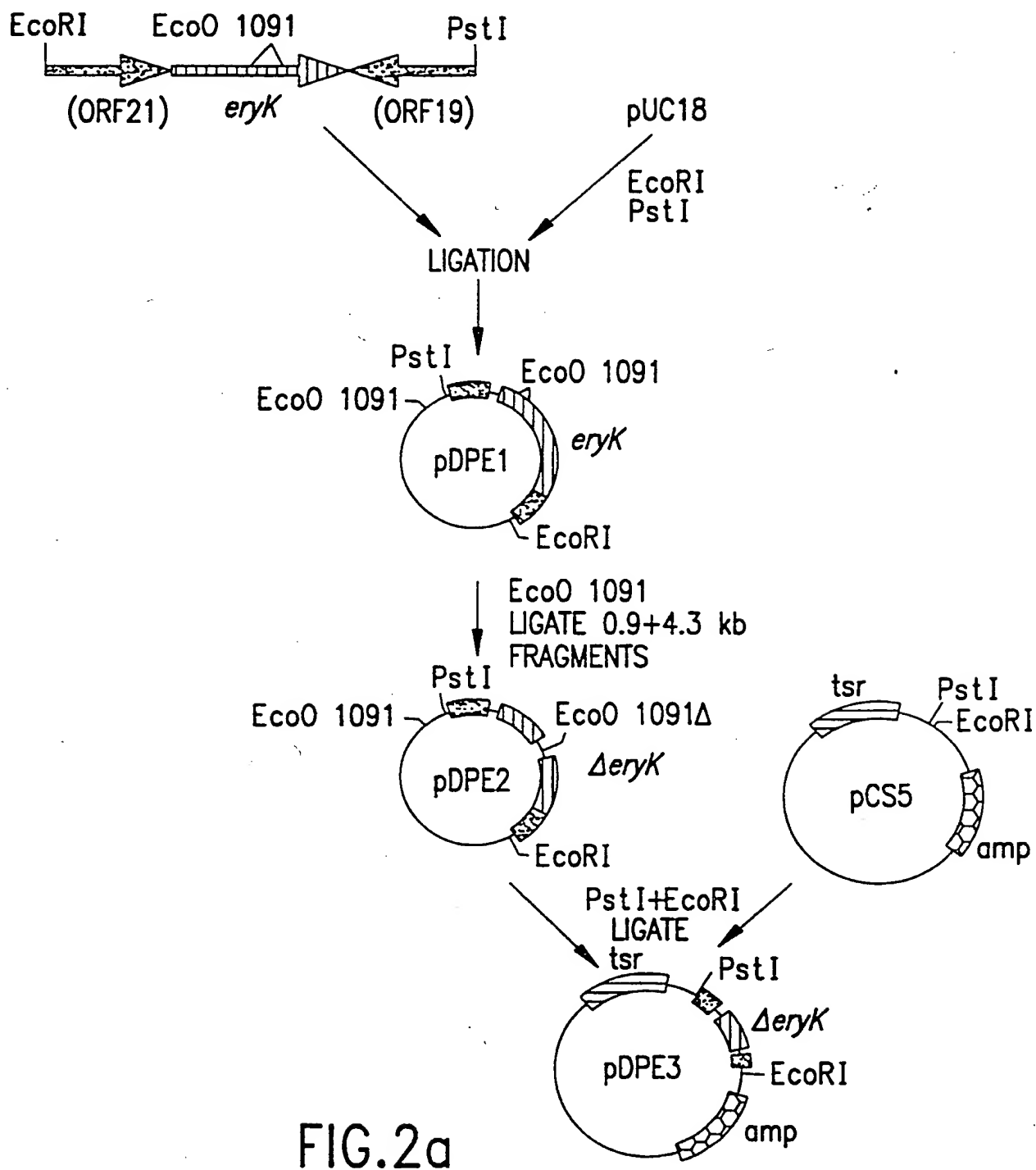


FIG.1

2/18



3/18

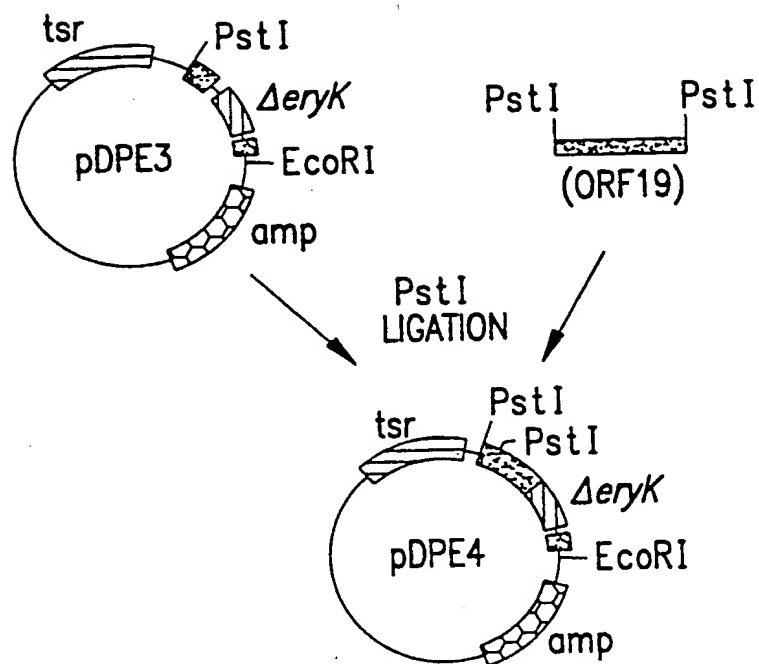


FIG.2b

4/18

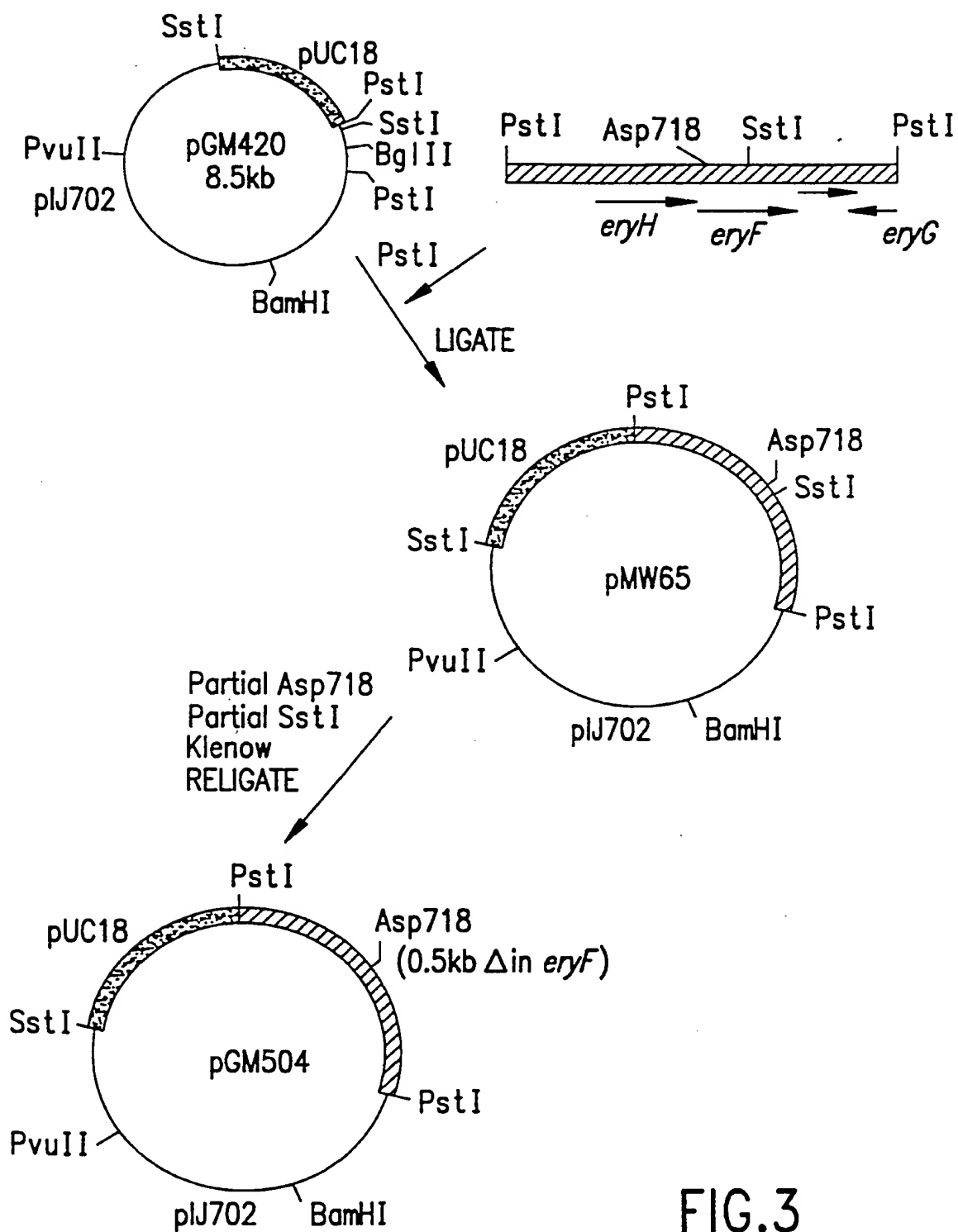


FIG.3

5/18

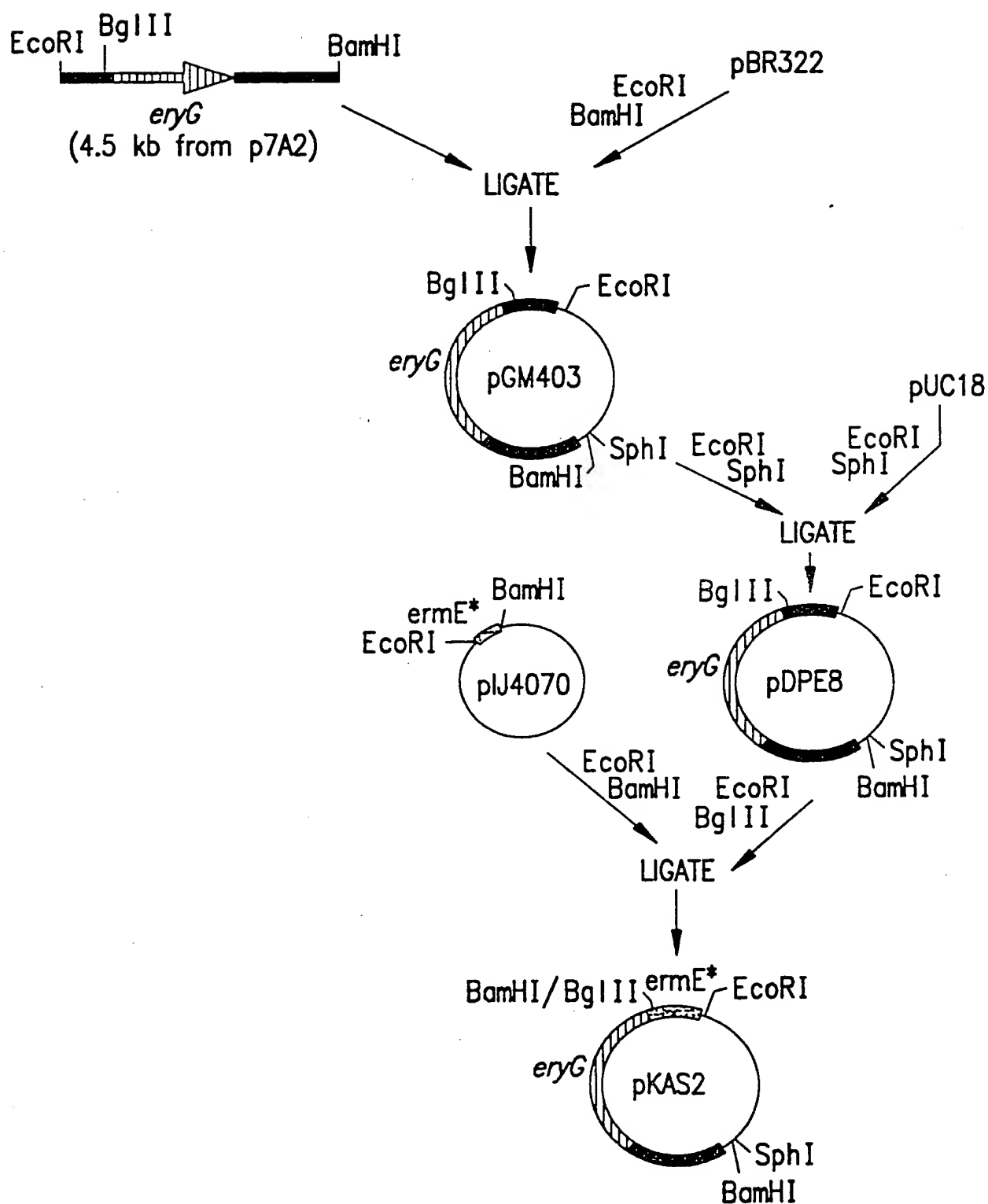


FIG.4a

6/18

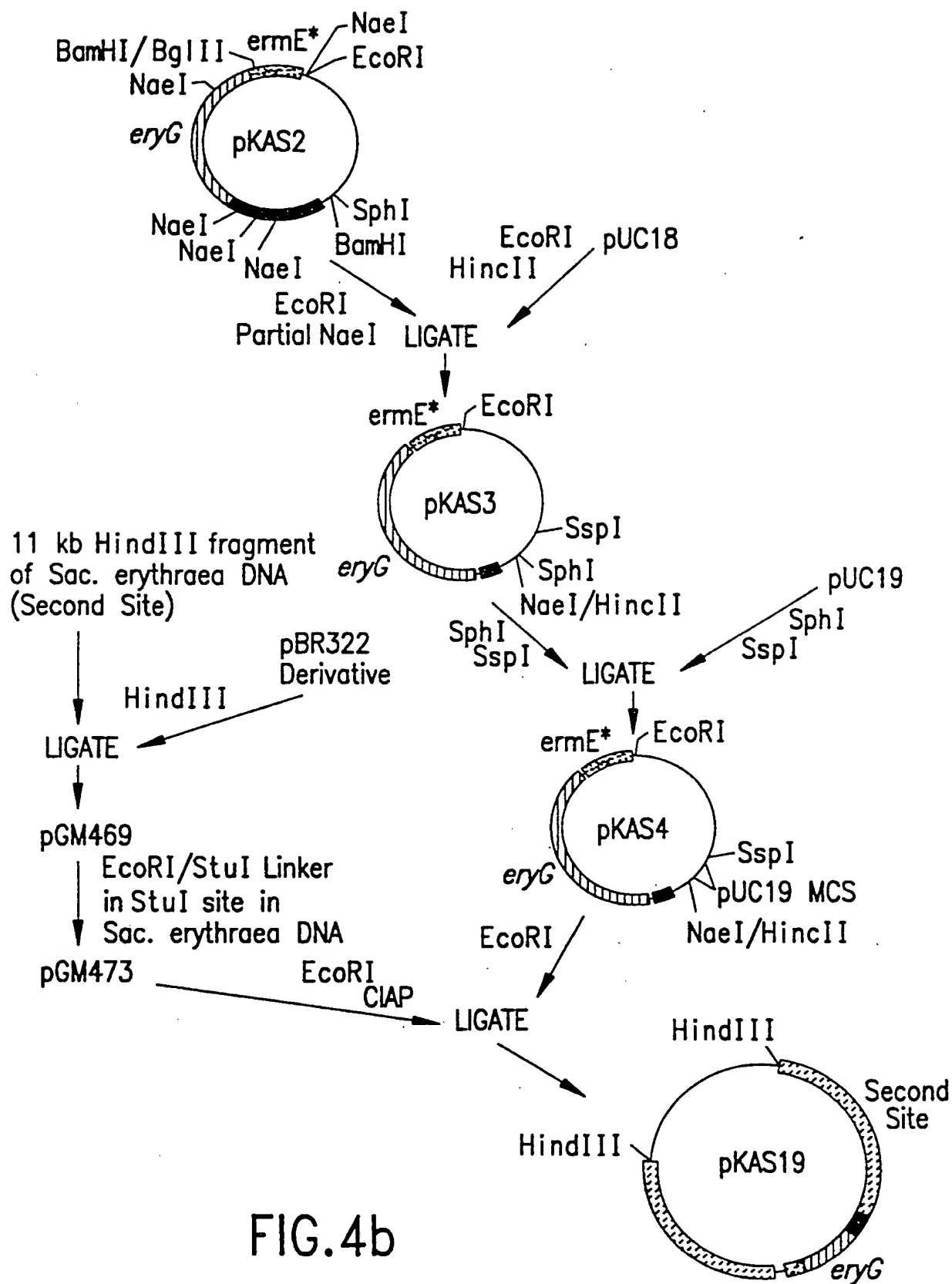


FIG.4b

7/18

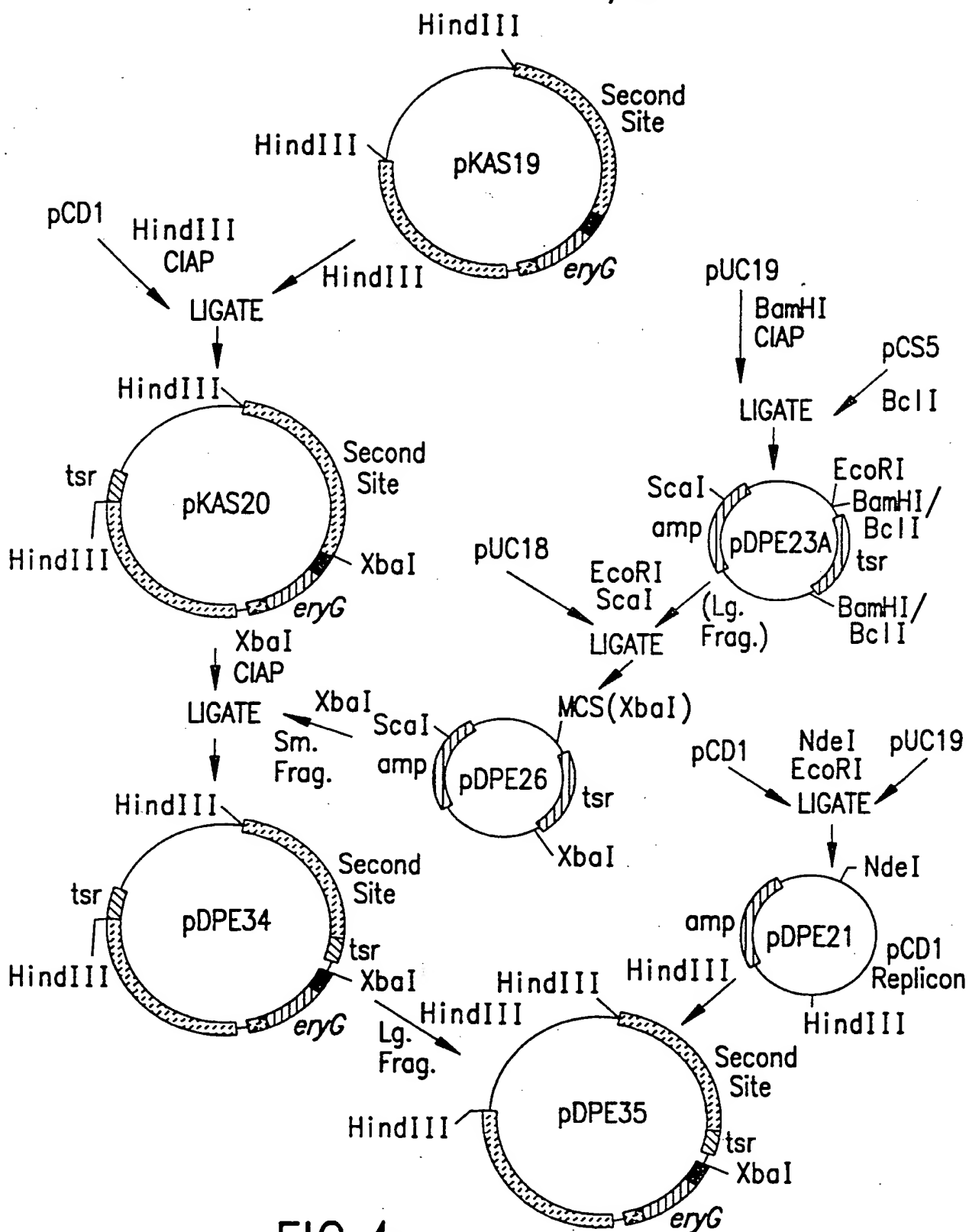


FIG.4c

8/18

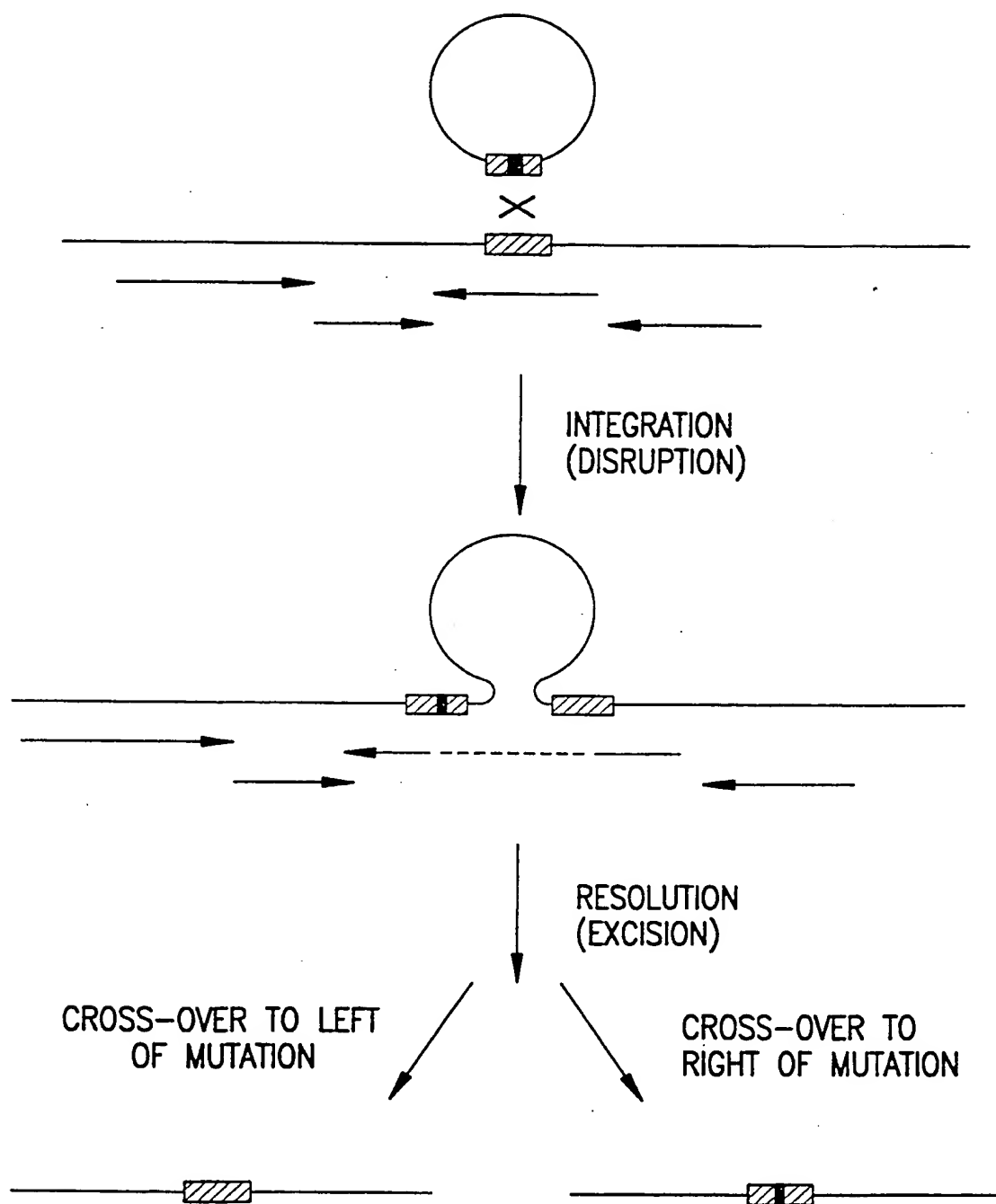


FIG.5

9/18

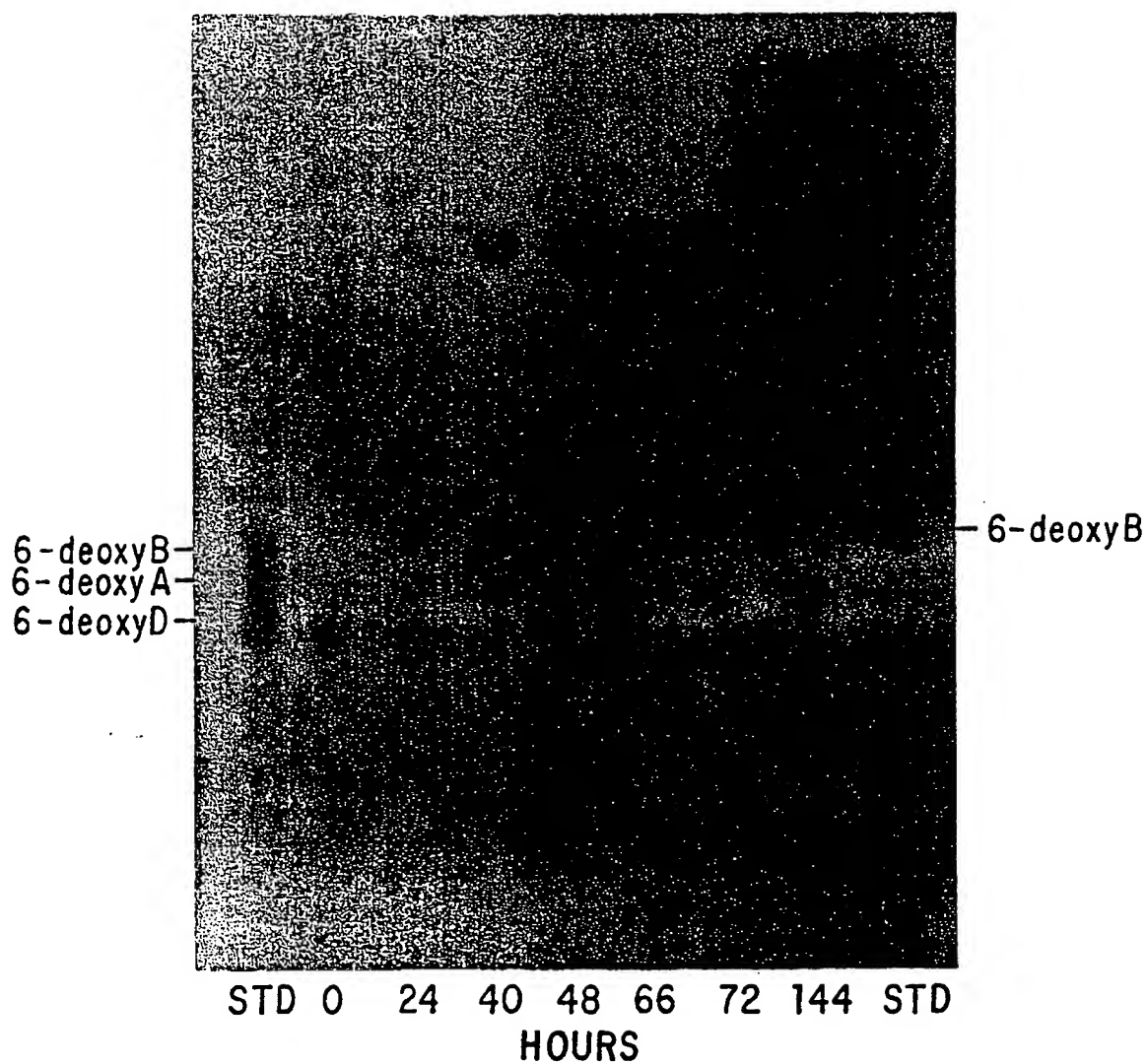


FIG.6

10/18

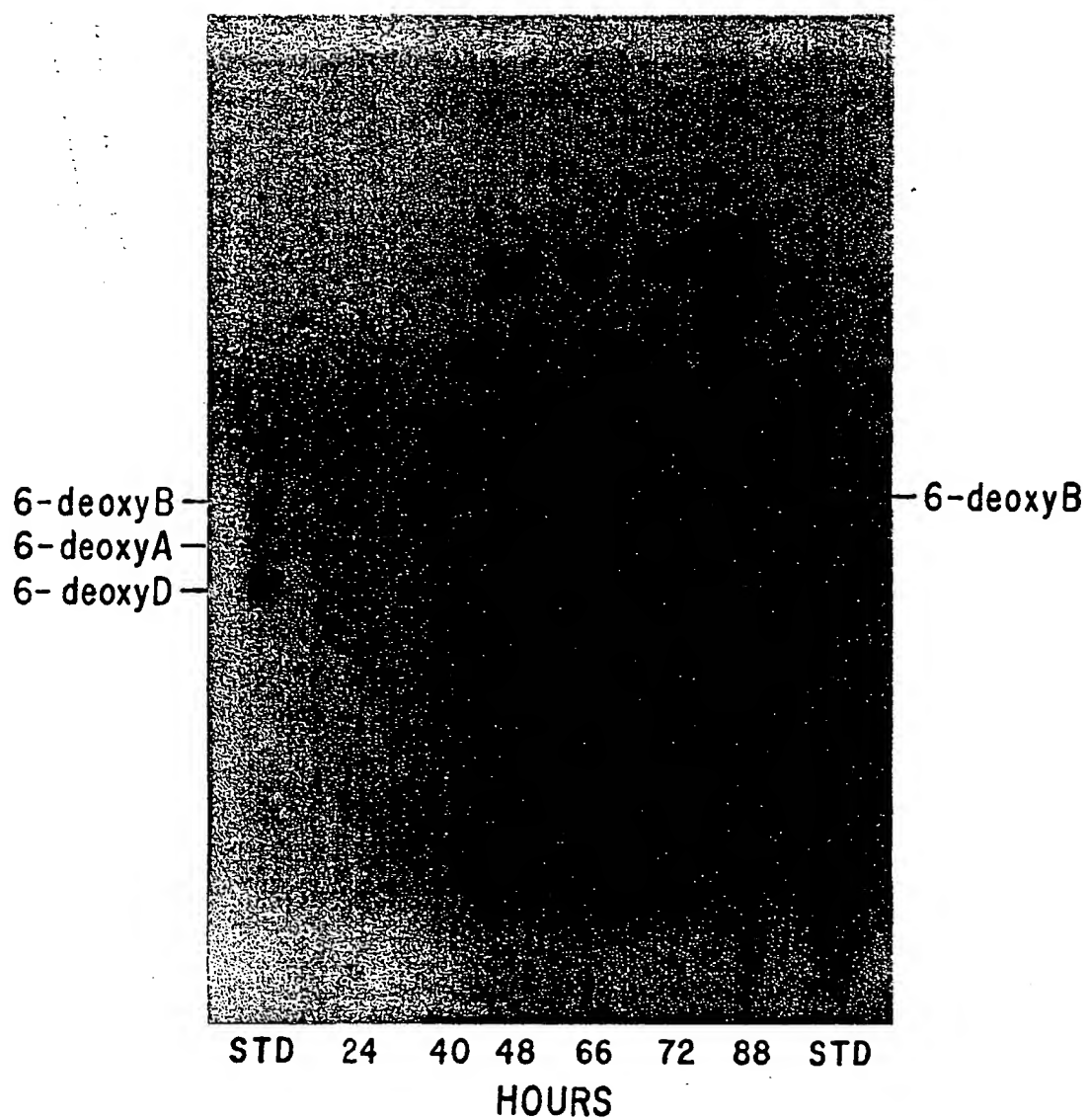


FIG.7

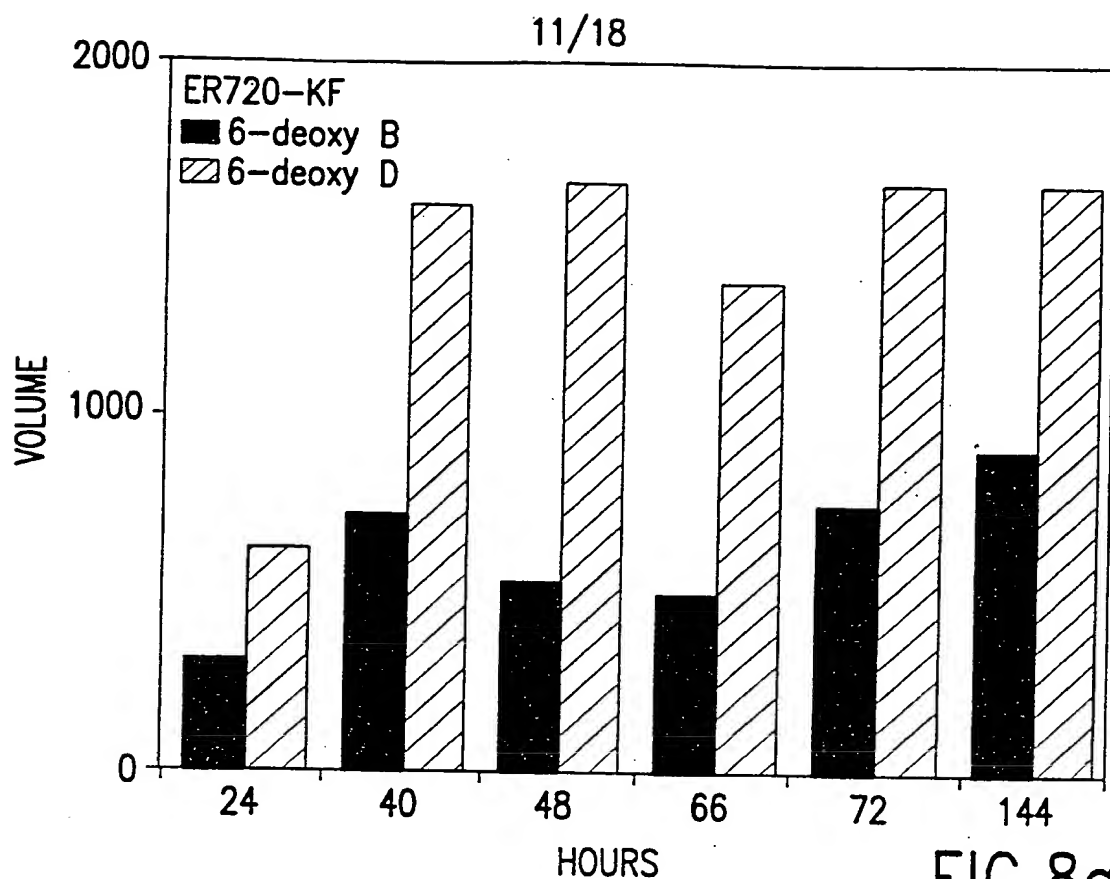


FIG. 8a

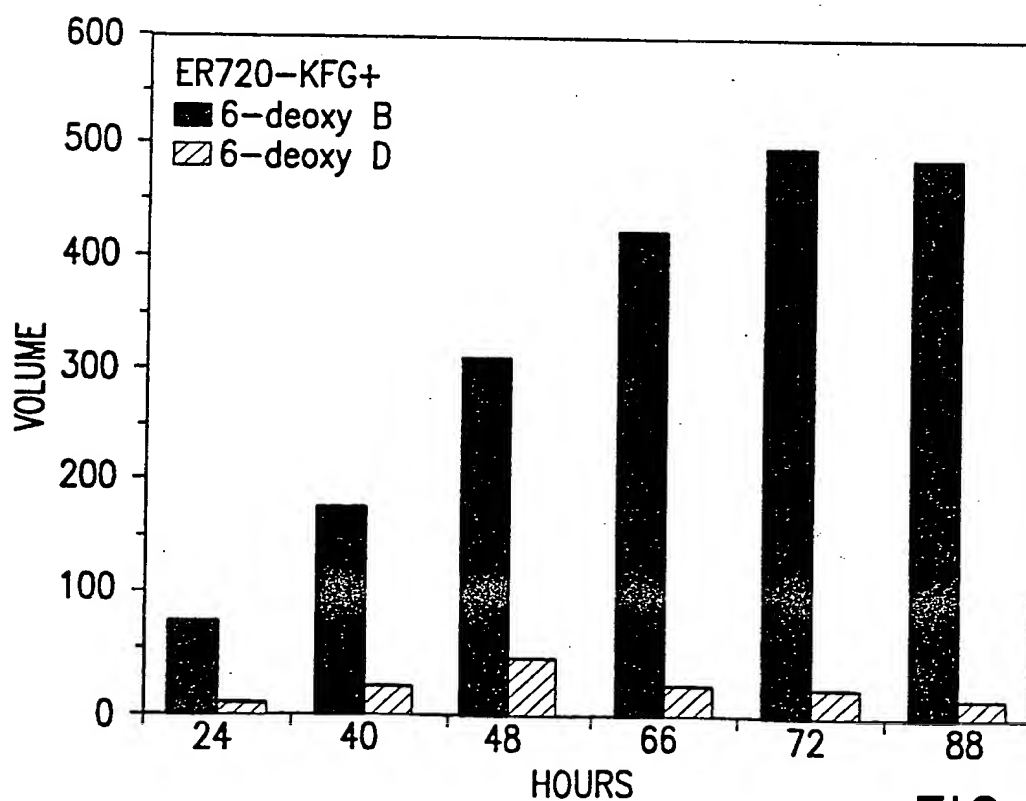


FIG. 8b

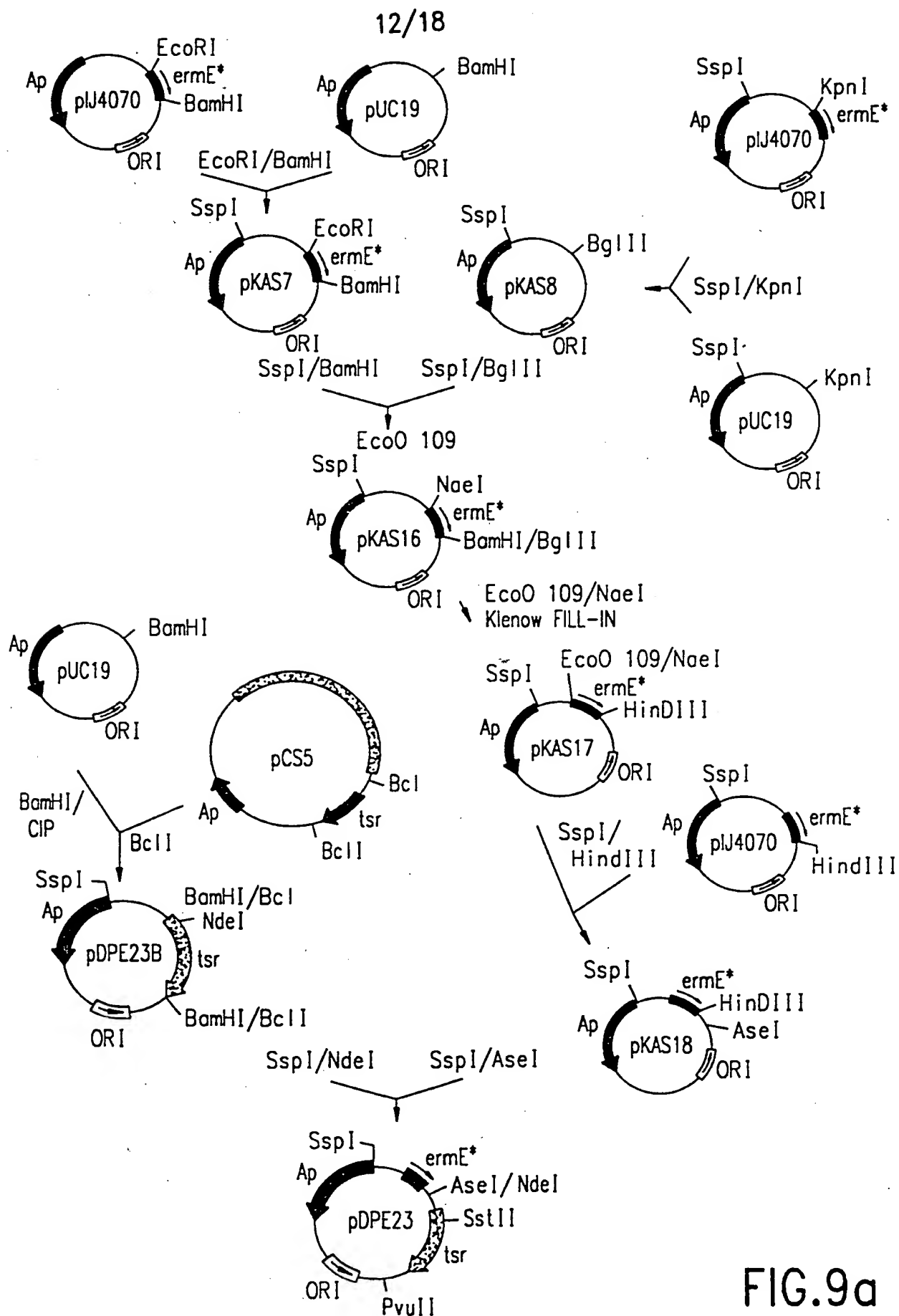


FIG.9a

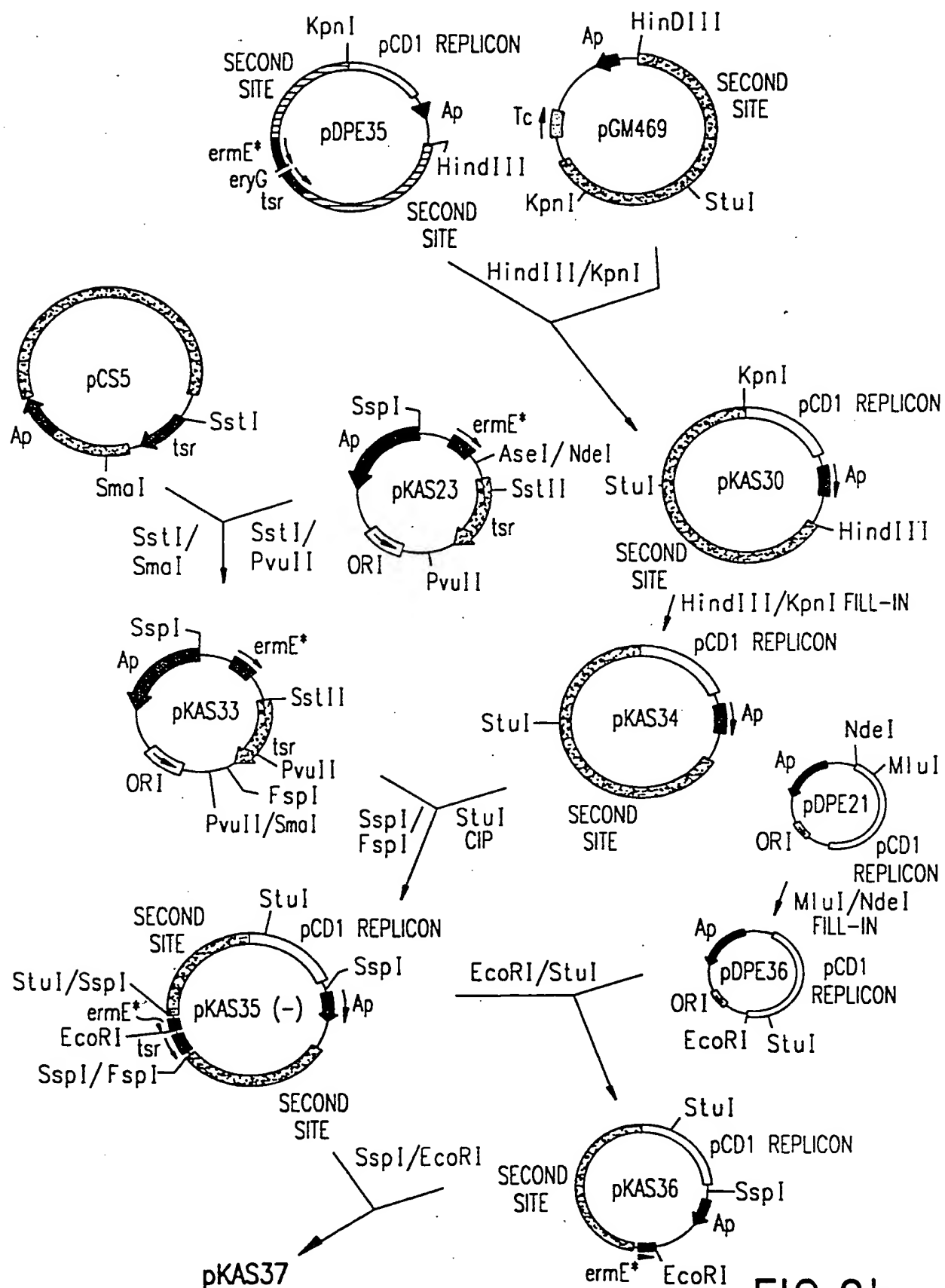


FIG.9b

14/18

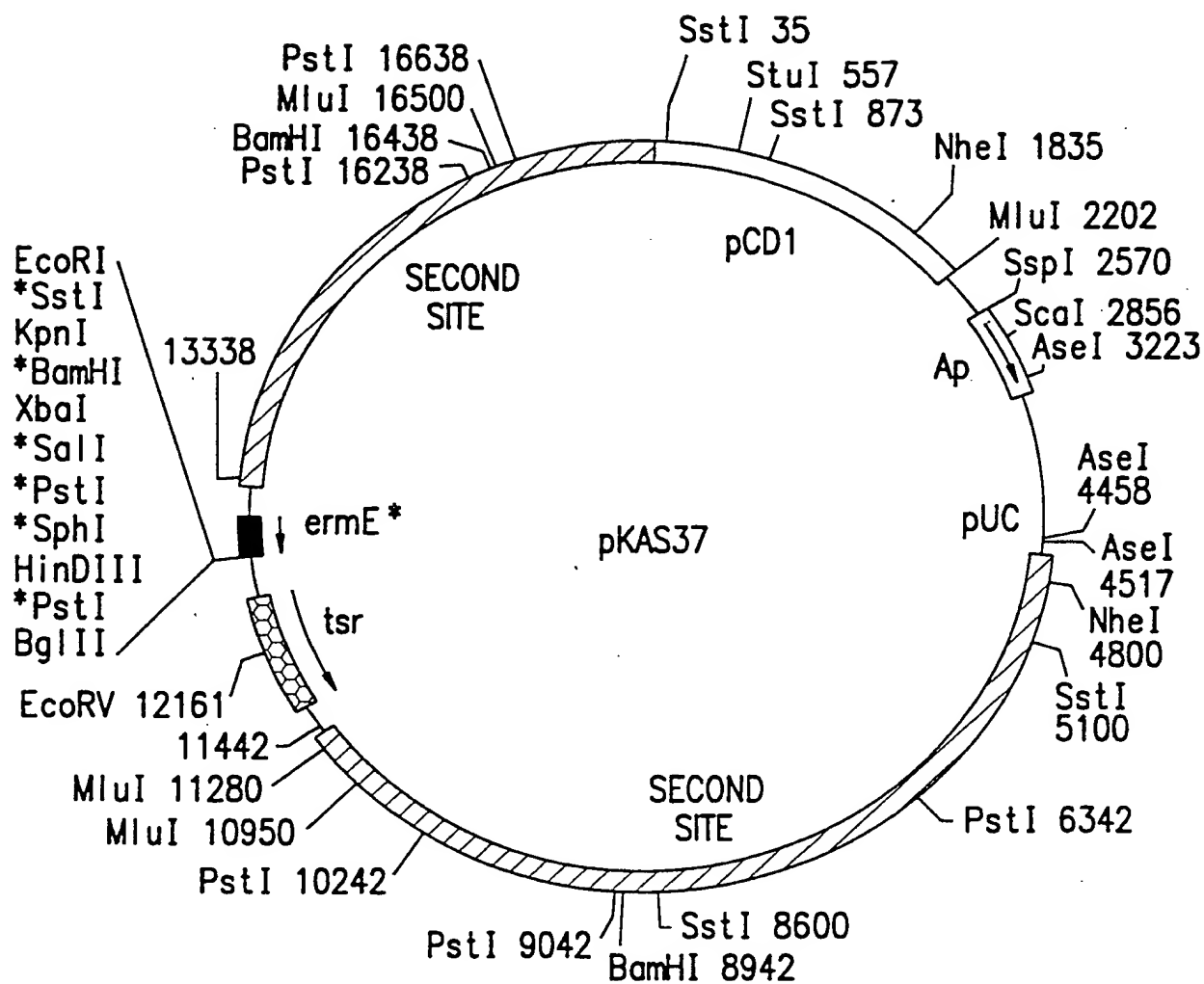


FIG.10

15/18

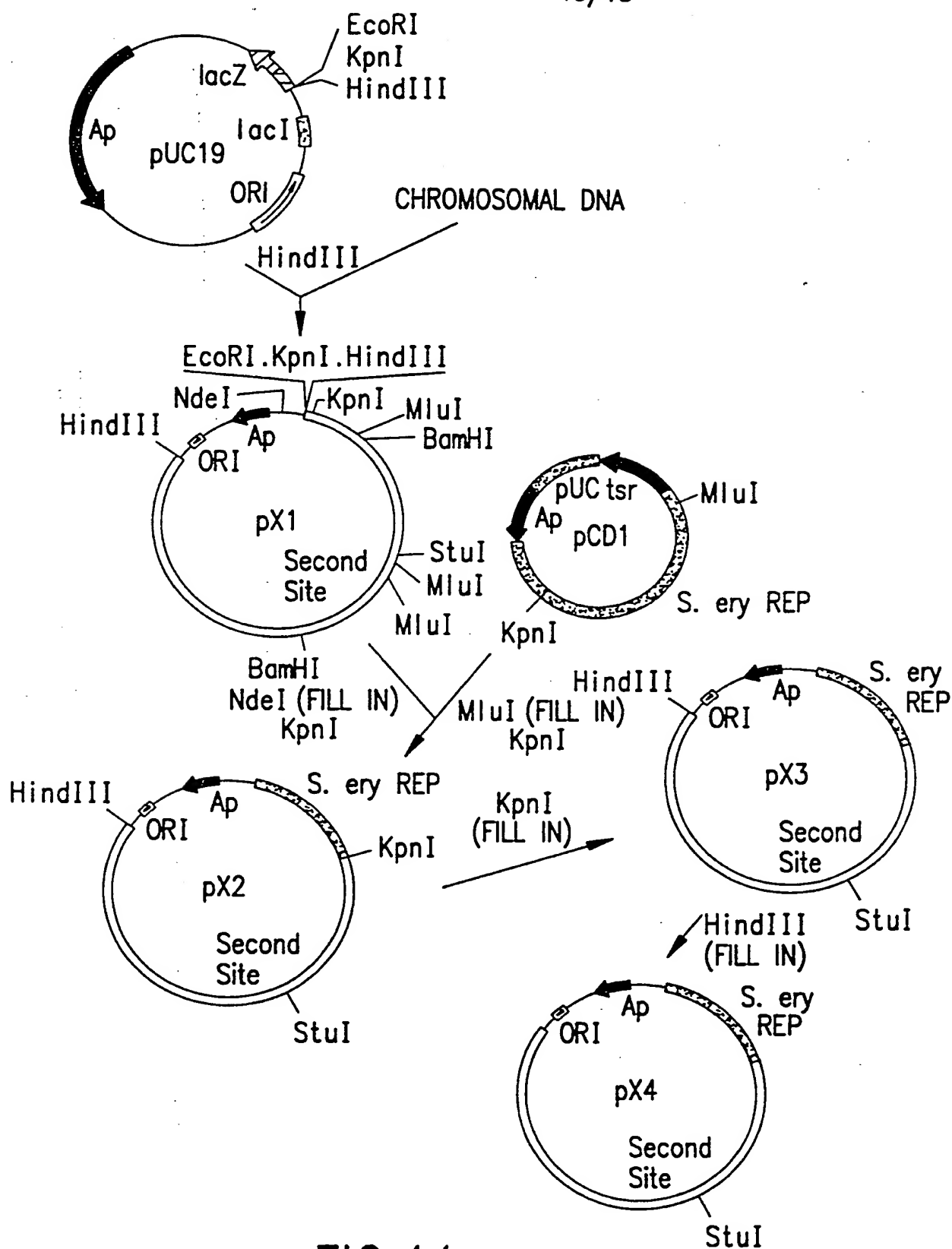


FIG.11a

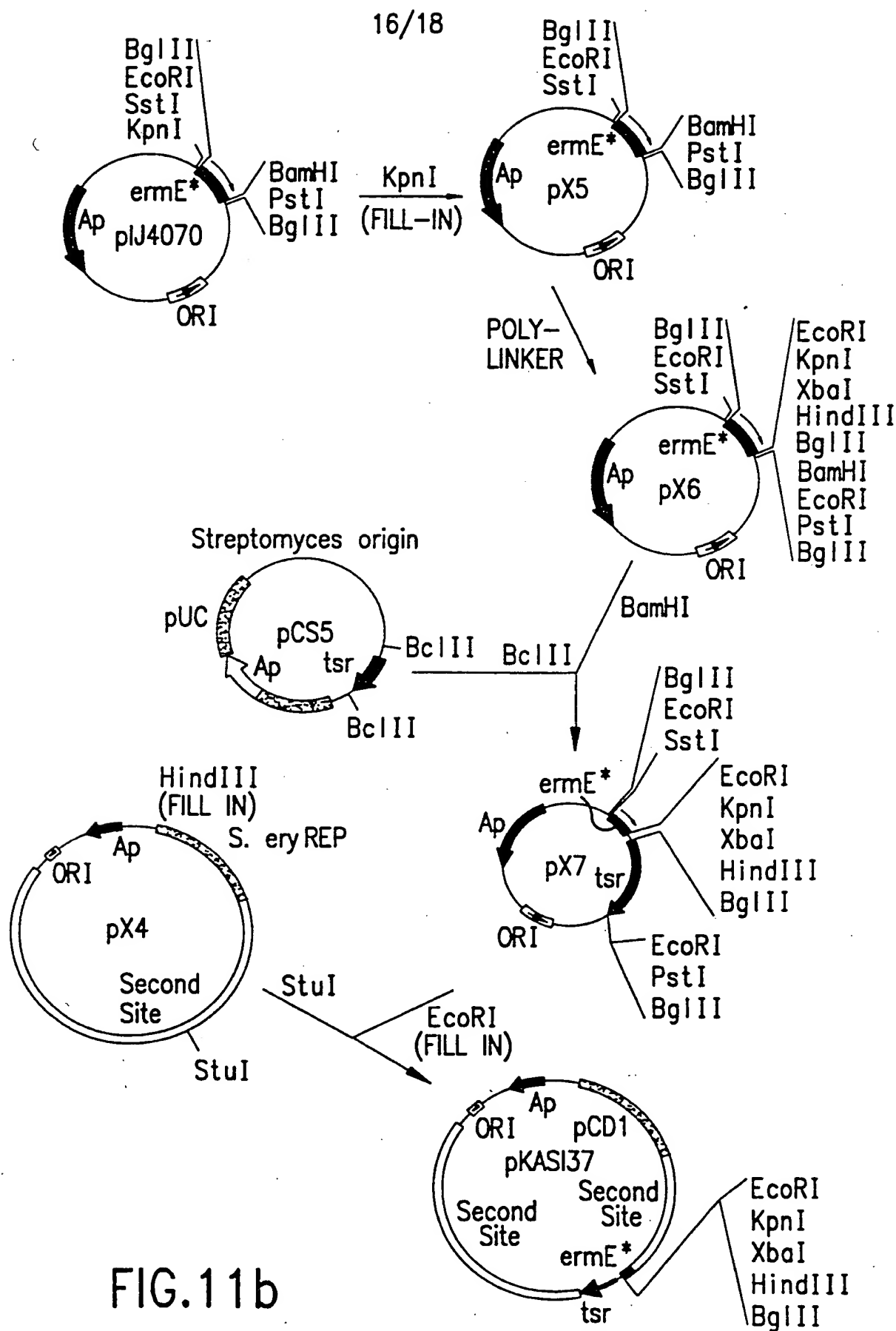


FIG.11b

SUBSTITUTE SHEET (RULE 26)

17/18

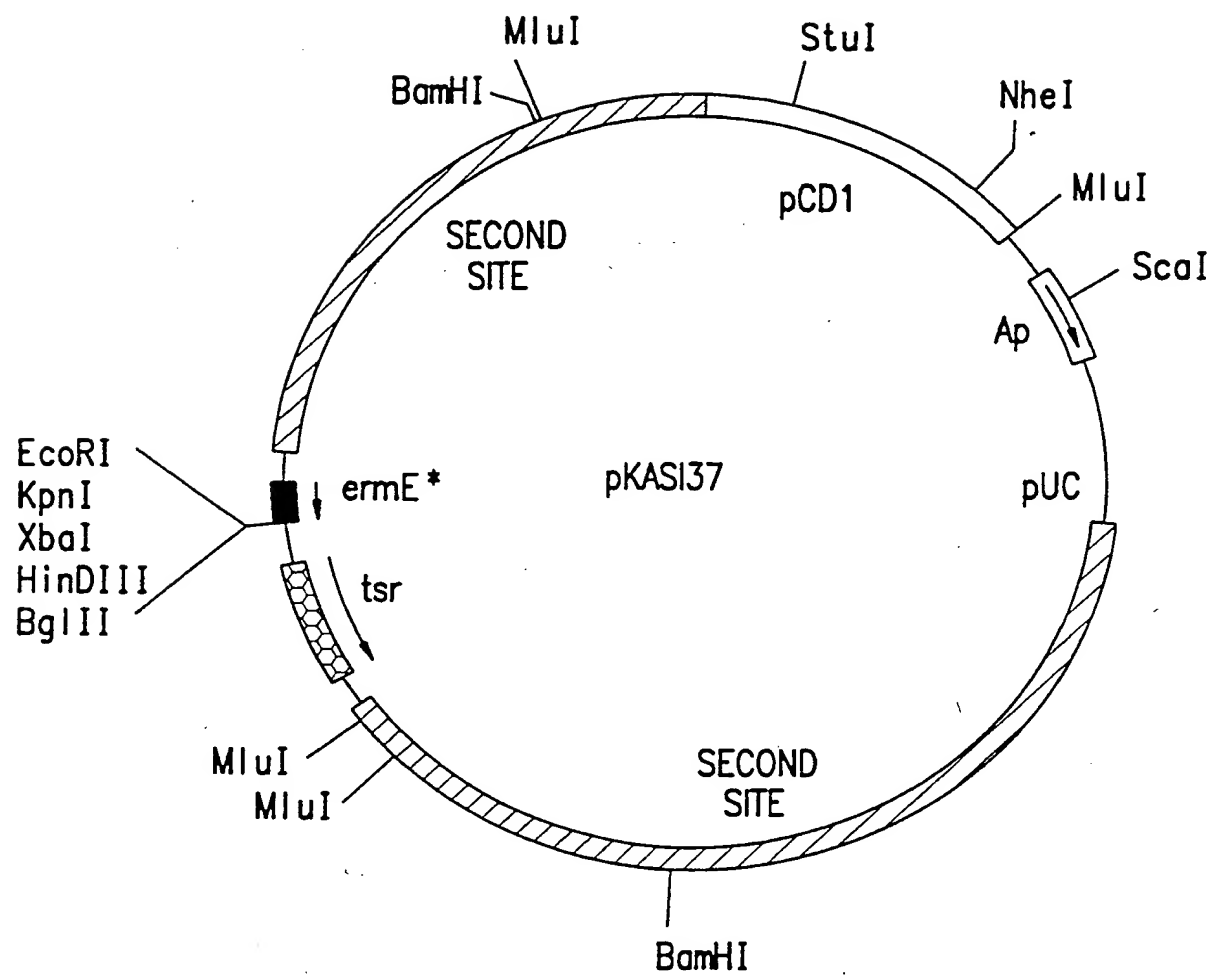


FIG.12

18/18

GAGCGACCAC AGGTGGGCCC GGATGTTGCA GCCTTGGTCG GGGTAGTCGA TCGGATTTCG 60
GAACAGTGCC ACGGCTGTGG TGTTCGAAGG TGAAGTCTT GAGCTGCTGG TGCCACCGGA 120
TTGCTTGCTC CAGCGAGACC GCGTTGCCGT TGACGAAGGC CAACGCGTCA AACACCGCCT 180
GGGAGTGCTC GGGTCGCAGT TTCTTCAAGT CATCGCTGAG AATCCCGGCA CCGAGCGTGA 240
TAGGCATCCT GCACCGCCCC ACACGGCGCG GAGATTGCGG TCCAGGCCCG GCAACATACC 300
AGCGCTTCGT CGAACTCGTC CGCCTCGACG TGGGCCCGCA GTTGTTCCGC GAACACTGCG 360
CAGTTCGGAG CAGCTTCTGG CCCAGGGCTT GCGACAACCT TGGGTGGGGT GTGCGCGGGG 420
TTGGTGCTGA AGTCGTTGCG GAAACCCAGC ATCGTCAGAG CGTGGTCGAA CTGTGCTGGA 480
CTGAGGTGCT CAGACAGCAC ACGAATCCAG CTCCCTGCCG GTGTGCTGCC AGAAGGGGAC 540
CGCGAGGCCC GCGGAATCTC CGCCGGATCG CCCC GAAGCC GACCCAGCTC ACGCAACACC 600
GAATCGGTGT CCGGCCGAGG TGACCGTGTG CCCGACCCGG AGCCGGGAGC ACGCCGCGCA 660
CTGGGCCTCC TCGGTTGTGT GTGTGAGATC GTCGTTCTC GAATTTAAGC AAGCCGGCGA 720
TGAAC TTCG CCGGCGCGCG GACAACGTCG TCACATCACC GTCCGCCCCG ACGCCAGAAG 780
CCGAGCCAGC CCCC GCACTG CGGCCCGAAC GGAACCTCCT CGGAAGTTAC GCCGGAGCTG 840
CCCGGTGCCG CCGTGGTCAG GAAAGCCTGC GCGTGCTGAG GGAGCCGTCC ATGTTGATAA 900
TTATTATCTC AGATGAC 917

FIG.13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/12850

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/76 C12N15/90 C12N9/10 C12P19/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,96 00282 (MERCK & CO.,INC.) 4 January 1996	1,3,5
P,Y	see the whole document	4
Y	<p>BIOTECHNOLOGY LETTERS, vol. 15, no. 2, February 1993, pages 105-110, XP000608366 HÄNEL, F., ET AL.: "Stimulation of Erythromycin A Yield by Integration of a Chromosomal DNA Fragment including the eryC1 Gene into the Chromosome of Saccharopolyspora erythraea" see the whole document</p> <p style="text-align: center;">-/-</p>	1,4,10, 12-14, 16,17

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

5 November 1996

Date of mailing of the international search report

15. 11. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Holtorf, S

INTERNATIONAL SEARCH REPORT

 Inter. Appl. No.
 PCT/US 96/12850

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BACTERIOLOGY, vol. 172, no. 5, May 1990, pages 2541-2546, XP000608716 PAULUS, T. ET AL.: "Mutation and Cloning of eryG, the Structural Gene for Erythromycin O-Methyltransferase from Saccharopolyspora erythraea, and Expression of eryG in Escherichia coli" see the whole document ---	1,4,9-17
Y	WO,A,91 16334 (ABBOTT LABORATORIES) 31 October 1991 see the whole document ---	9,11,15
A	WO,A,93 20215 (BOEHRINGER INGELHEIM GMBH) 14 October 1993 see the whole document ---	1-3
A	GENE, vol. 75, 1989, pages 235-241, XP002017374 WEBER, J. M., ET AL.: "Identification of a Gene required for the Terminal Step in Erythromycin A Biosynthesis in Saccharopolyspora erythraea (Streptomyces erythreus)" see the whole document ---	1-17
A	SCIENCE, vol. 252, no. 5002, April 1991, pages 114-117, XP002017375 WEBER, J.M., ET AL.: "An Erythromycin Derivative Produced By Targeted Gene Disruption in Saccharopolyspora erythraea" see the whole document ---	1-17
A	JOURNAL OF BACTERIOLOGY, vol. 175, no. 1, January 1993, pages 182-189, XP000608396 STASSI, D., ET AL.: "Identification of a Saccharopolyspora erythraea Gene Required for the Final Hydroxylation Step in Erythromycin Biosynthesis" see the whole document ---	1-17
A	BIOTECHNOLOGY, vol. 8, February 1990, pages 115-121, XP000087713 CHATER, K.F.: "The Improving Prospects for the Yield Increase by Genetic Engineering in Antibiotic-Producing Streptomyces" see page 119, right-hand column, line 10-50 --- -/--	1-17

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/12850

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DONADIO, S. ET AL .: "Recent Developments in the genetics of erythromycin formation. in: Industrial Microorganisms: Basic and Applied Molecular Genetics. Edited by R.H. Baltz, G.D. Hegemann, P.L. Skatrud; pp. 257-265"</p> <p>1993 , AMERICAN SOCIETY FOR MICROBIOLOGY , WASHINGTON, DC 20005 XP000607676</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	1-17
A	<p>MOLECULAR AND GENERAL GENETICS, vol. 203, 1986, pages 79-88, XP000608705</p> <p>LYDIATE, D.J. ET AL.: "A 2.6 kb DNA sequence of Streptomyces coelicolor A3(2) which functions as a transposable element"</p> <p>see the whole document</p> <p style="text-align: center;">-----</p>	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/12850

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9600282	04-01-96	AU-A- 2866795	19-01-96
WO-A-9116334	31-10-91	US-A- 5141926	25-08-92
		CA-A- 2080583	19-10-91
		EP-A- 0525083	03-02-93
		JP-T- 5504890	29-07-93
WO-A-9320215	14-10-93	DE-A- 4211517	14-10-93
		EP-A- 0640139	01-03-95
		JP-T- 7501708	23-02-95